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Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant

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The ability of the *Lactobacillus acidophilus* RC14 biosurfactant 'surlactin' to inhibit the initial adhesion of various uropathogenic bacteria and two yeast strains to silicone rubber was investigated in a parallel-plate flow chamber in filter-sterilised pooled human urine. A parallel-plate flow chamber with a silicone rubber bottom plate was filled with a 1.0 mg/ml biosurfactant solution for adsorption overnight (18 h). Subsequently, the adhesion of the bacterial or yeast cells from a urine suspension under low flow (shear rate 15 s^{-1}) was followed *in situ* by automated image analysis. Control tests were with untreated silicone rubber. Initial deposition rates and numbers of adhering cells after 4 h of flow were determined. Surlactin layers caused a marked inhibition of the initial deposition rates and adhesion numbers after 4 h for the majority of the bacteria (11 of 15 strains tested) and this inhibition was particularly effective against *Enterococcus faecalis, Escherichia coli* and *Staphylococcus epidermidis*. Although the initial deposition rates of the two *Candida albicans* strains were reduced by *c.* 50% in comparison with the controls, the numbers of yeast cells adhering after 4 h were similar.

Introduction

Catheterisation of the urinary tract remains a major cause of hospital-acquired infection [1]. Despite the use of aseptic insertion techniques and the conversion from open to closed sterile drainage systems in the 1960s [2], the incidence of bacteriuria is 5–10% per day during the first week of catheterisation [3, 4] and bacteriuria will inevitably develop in almost 100% of patients on long-term catheterisation (>30 days) [5].

In the first week, catheterised patients usually become bacteriuric with a single species [3, 4]. *Escherichia coli* is responsible for about a quarter of these early infections [6, 7], in contrast to urinary tract infection in non-catheterised patients of which up to 80% can be caused by *E. coli* [7]. Other common species isolated from bacteriuric patients on short-term catheterisation are *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus epidermidis, Enterococcus faecalis* and *Proteus mirabilis* [6]. The variety of infecting organisms increases with the time the catheter remains in place, and in chronically catheterised patients, bacteriuria is polymicrobial with sometimes 6–8 different species, each present in high concentration ($\geqslant 10^5$ cfu/ml) [8]; the proportion of infections with *E. coli* becomes smaller (14%) [9] and other gram-negative organisms such as *Providencia stuartii, P. aeruginosa, Pr. mirabilis* and *K. pneumoniae* gain importance [9, 10]. *Candida* spp. may also be cultured, usually following antibacterial therapy [3], with *C. albicans* as the commonest isolate [11].

Bacterial biofilms, particularly the complex mixed biofilms, are difficult to eradicate, as bacteria growing within the protecting glycocalyx of a biofilm are relatively resistant to antibiotic therapy and host defence mechanisms [12]. To treat difficult or recurrent urogenital infections some clinicians have attempted to re-establish the healthy physiological ecology of the female urogenital tract by recolonisation with probiotic lactobacilli, the major indigenous

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organisms, or by the use of oestrogen to stimulate the re-emergence of this flora. Oral or topical administration of oestrogens to post-menopausal women with a history of recurrent urinary tract infection, for example, resulted in an increase in the number of women with vaginal lactobacilli and a decrease in the number with uropathogens, possibly by a lowering of the vaginal pH [13, 14]. Vaginal instillation with viable lactobacilli has been shown to be effective in the prevention of recurrent urinary tract infection in women, although placebo-controlled trials are still required [15, 16].

In-vitro studies have demonstrated that lactobacilli can displace adhering uropathogens from catheter materials such as silicone rubber [17, 18] and block adhesion of uropathogenic bacteria to human uroepithelial cells [19] and polymer substrata [17, 20]. Furthermore, lactobacilli can produce various antimicrobial compounds such as lactic acid, hydrogen peroxide and bacteriocins [21]. Recently, *Lactobacillus* strains that produce biosurfactants have been described; these inhibited the initial adhesion of a uropathogenic *Ent. faecalis* strain to hydrophobic and hydrophilic substrata in the presence of phosphate-buffered saline or urine, as studied in a parallel-plate flow chamber [22, 23]. Biosurfactants are microbial compounds that have some effect on interfaces, most notably on the surface tension of liquid-vapor interfaces. The release and composition of biosurfactants from lactobacilli has been shown to be growth-dependent and to vary with each strain [24]. All 15 *Lactobacillus* isolates tested in an earlier study released biosurfactants, but only some strains could effectively minimise initial enterococcal adhesion [22]. The stationary growth phase biosurfactants from *L. acidophilus* RC14 and *L. fermentum* B54 caused a marked decrease in the initial deposition rate of *Ent. faecalis*, whereas those released by *L. casei* subsp. *rhamnosus* 36 and ATCC 7469 did not [22]. Identification of the multicomponent biosurfactants showed that they contained protein and polysaccharides which were possibly bound to phosphate groups. Neither Fourier transform nor X-ray photoelectron spectroscopy suggested the presence of lipoteichoic acid. The stationary phase biosurfactants from *L. acidophilus* RC14 and *L. fermentum* B54 were the most proteinaceous (28–30% protein by the BioRad protein assay) and have been named 'surlactin' [24]. Amino-acid analysis of surlactin released by *L. acidophilus* RC14 indicated a high molar content of alanine in mid-exponential growth phase (40%), which fell to 11% in stationary growth phase. SDS-PAGE of stationary growth phase RC14 biosurfactants showed various bands ranging in mol. wt from 14.4 to >94 kDa.

The aim of this study was to investigate the potential of surlactin from *L. acidophilus* RC14 as an anti-adhesive coating for catheter materials such as silicone rubber.

Materials and methods

Strains and culture conditions

Seventeen uropathogenic organisms were used in the study: *E. coli* (2 isolates), *K. pneumoniae* (2), *Pr. mirabilis* (2), *Prov. stuartii* (2), *P. aeruginosa* (2), *S. epidermidis* (2), *Ent. faecalis* (3) and *C. albicans* (2). All strains were clinical isolates from urinary tract infections apart from one *P. aeruginosa* strain (ATCC 10145). All bacterial strains were stored in growth medium containing dimethyl sulphoxide 7% v/v at −60°C. Growth media (all supplied by Oxoid) included blood agar and Brain Heart Infusion (BHI) broth for *E. coli* and *Ent. faecalis*; nutrient broth (NB) for *K. pneumoniae, Pr. mirabilis* and *P. aeruginosa*; blood agar and NB broth for *Prov. stuartii*; blood agar and Tryptone Soya Broth for *S. epidermidis*. *Candida* strains were stored on silica gel 60 at −20°C and cultured on BHI. From frozen stock, the bacteria and yeasts were streaked on agar slants and incubated aerobically at 37°C overnight. Precultures were grown under the same conditions by inoculating 10 ml of broth with a colony from the plate. A 24-h preculture was used to prepare 200 ml of an 18-h stationary culture. The cells were harvested by centrifugation (4070 *g*, 5 min, 10°C), washed twice in demineralised water, and, if necessary, sonicated three times for 10 s at 30 W on ice with a Vibra Cell 375 (Sonics and Materials, Danbury, CT, USA) to break aggregates. After counting in a Bürker-Türk counting chamber, the bacteria and yeasts were diluted in 200 ml of urine to a final density of 3×10^8 cells/ml and 3×10^6 cells/ml, respectively, for adhesion assays.

L. acidophilus RC14, an isolate from the urogenital tract of a healthy woman [25], was stored in MRS broth [26], (Merck, Darmstadt, Germany) containing dimethyl sulphoxide 7% v/v at −60°C. From frozen stock, MRS agar slants were inoculated and incubated at 37°C in an atmosphere containing CO_2 5% to obtain cultures. From a 24-h preculture in 15 ml of MRS broth, 600 ml of an 18-h stationary culture was prepared for biosurfactant isolation.

Isolation of biosurfactant

The stationary cells of *L. acidophilus* RC14 were harvested by centrifugation (10 000 *g*, 5 min, 10°C), washed twice in demineralised water, and resuspended in 100 ml of phosphate-buffered saline (10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). The lactobacilli were left at room temperature for biosurfactant release, with gentle stirring to keep them suspended. After 2 h, the supernate containing the biosurfactant was separated from the cells by centrifugation and filtered through a 0.22-μm pore-size filter (Millipore, Bedford, MA, USA). The filtrate was then dialysed against demineralised water at 4°C in a Spectrapor membrane tube (mol. wt cut off 6000–8000; Spectrum Medical Industries, CA, USA), freeze-dried, and stored at −20°C.

Urine

Urine was collected from 16 healthy adult volunteers (12 male; 4 female) over two consecutive days. After removal of sediment by centrifugation (10 000 *g*, 10 min, 10°C), the urine was pooled, divided into 400-ml portions, and stored at −20°C. Before use, the urine was warmed to room temperature and sterilised by filtration successively through a 5.0-μm and a 0.45-μm pore-size filter (Millipore). The pH of the pooled urine was 6.5–6.7.

Substratum surface

A silicone rubber substratum (Silastic® medical grade silicone rubber, Nusil, Belgium) 5.5 cm × 3.8 cm was cleaned ultrasonically in a RBS surfactant solution 2% in water (Omnilabo International BV, The Netherlands) for 5 min, and rinsed thoroughly with warm tap water, methanol and demineralised water. It was fixed to a polymethyl methacrylate plate with double-sided sticky tape to make its surface more rigid. After cleaning, the silicone rubber had a water contact angle of 110°, indicative of a hydrophobic surface. Its surface roughness was $R_A = 0.4 \pm 0.1$ μm. The silicone rubber surface was placed on the bottom of a flow chamber, separated from an upper glass plate of equal dimensions by two Teflon spacers 0.06 cm thick.

Adhesion assay

Deposition of uropathogenic bacteria and yeasts to silicone rubber with and without an adsorbed layer of surlactin was studied in a parallel-plate flow chamber. Automated image analysis allowed in-situ observation of bacterial and yeast cell adhesion over a microscopic field of view covering 0.011 mm^2 and 0.167 mm^2, respectively, as described previously [22].

A parallel-plate flow chamber was filled with a surlactin solution of 1.0 mg/ml in phosphate-buffered saline for overnight adsorption (18 h) at 4°C. Subsequently, the biosurfactant solution was drained from the flow chamber and a bacterial or yeast suspension in urine (3×10^8 cells/ml and 3×10^6 cells/ml, respectively) was allowed to flow through the system at room temperature. Experiments on the prepared biosurfactant layers and controls on clean silicone rubber were performed simultaneously with the same batch of bacteria or yeasts. A pulse-free flow (0.034 ml/s) was created by hydrostatic pressure and the suspension was recirculated by a Multiperpex 2115 peristaltic pump (Pharmacia LKB Biotechnology, Uppsala, Sweden), maintaining a constant shear rate of 15 s^{-1}. Based on the estimated daily urine production and internal catheter diameter, this shear rate is similar to that found at the luminal surface of a urinary catheter, and corresponds to a Reynolds number of 1, well within the laminar flow regimen. During the experiment, images were obtained and stored in the computer. From the initial, linear increase in the number of adhering bacteria or yeasts/unit area with time, the initial deposition rate was calculated by a linear, least-squares fitting procedure. Experiments were performed in triplicate for some strains with results corresponding to within 20%.

Results

Table 1 shows the initial deposition rates and numbers of adherent bacteria or yeasts after 4 h for various uropathogens on clean silicone rubber (controls) and on silicone rubber with an adsorbed layer of the surlactin biosurfactant. For 11 of the 15 uropathogenic bacteria tested, both the initial deposition rates and the numbers of adherent bacteria after 4 h were markedly reduced by the surlactin layers. This inhibition was particularly strong and consistent for *Ent. faecalis, E. coli* and *S. epidermidis*. Moreover, the numbers of adherent *P. aeruginosa* after 4 h on surlactin-coated silicone rubber was reduced by almost 50% compared with the control, although the initial deposition rate was unaffected.

In contrast, adsorbed surlactin layers caused a decrease in the initial deposition rates of *K. pneumonia* 3a, *Prov. stuartii* UHL 5292 and *P. aeruginosa* AK1 and the two *C. albicans* strains, but had only a minor effect on the numbers of adherent organisms after 4 h.

Discussion

The ability of the *L. acidophilus* RC14 biosurfactant surlactin to inhibit the initial adhesion of uropathogenic bacteria and yeasts to silicone rubber was examined in a parallel-plate flow chamber in the presence of human urine. Silicone rubber with an adsorbed layer of surlactin showed a marked reduction in the initial adhesion for the majority of the bacteria, but did not affect the initial adhesion of two *C. albicans* strains. Agar diffusion experiments demonstrated that surlactin, up to a concentration of 1.0 mg/ml, did not inhibit growth of any of the pathogens (unpublished data).

The parallel-plate flow chamber provides excellent adhesion data compared to other methods involving 'dipping', 'slight rinsing', etc. However, the method requires a relatively large amount of fluid to coat the silicone rubber substrata, and this, coupled with the slow release of surlactin by lactobacilli, precluded the study of subfractions of surlactin. Recently, Reid *et al.* [27] isolated eight fractions from surlactin by HPLC size exclusion chromatography, of which four were surface active and only two were effective at reducing enterococcal adhesion in static adhesion assays. The glycoproteinaceous character of surlactin was confirmed by phenol-sulphuric acid analysis, and the inhibitory activities were resistant to trypsin, pepsin and heat treatment [27].

Table 1. Initial deposition rates and adhesion counts of various uropathogenic bacteria and yeasts after 4 h on silicone rubber with and without an adsorbed surlactin layer

Strain	Biosurfactant layer (−/+)	Initial deposition rate ($1/s/cm^2$)	Number after 4 h ($10^6/cm^2$)
C. albicans urine 1	−	101	1.1
	+	58	1.1
C. albicans urine 2	−	76	0.8
	+	38	0.7
E. coli 67	−	197	2.4
	+	17	0.5
E. coli Hu734	−	17	1.1
	+	0	0.2
Ent. faecalis 1131*	−	111	1.0
	+	0	0.04
Ent. faecalis 1396	−	47	0.3
	+	0	0.02
Ent. faecalis 4b	−	113	0.9
	+	0	0.02
K. pneumoniae 280	−	234	2.4
	+	0	0.2
K. pneumoniae 3a	−	152	0.9
	+	40	0.7
Pr. mirabilis 296*	−	163	1.9
	+	61	0.3
Pr. mirabilis 28cii*	−	160	1.0†
	+	64	0.5†
Prov. stuartii UHL 103	−	288	8.9
	+	122	4.0
Prov. stuartii UHL 5292	−	34	0.3
	+	7	0.2
P. aeruginosa AK1	−	648	11
	+	420	9.5
P. aeruginosa ATCC 10145	−	288	11
	+	365	6.1
S. epidermidis 3059*	−	95	1.8
	+	58	0.2
S. epidermidis 3081*	−	675	2.3
	+	110	0.4

Experiments and controls were performed simultaneously with the same batch of bacteria or yeasts.
*Triplicate experiments, corresponding within 20%.
†Number of adhering bacteria after 2 h.

Various physiological roles have been suggested for microbial surfactants. Biosurfactants can enable microorganisms to grow on hydrocarbons by stimulating adhesion to the organic substrate or emulsification of water-insoluble carbon sources [28]. Some biosurfactants have been shown to have antiobiotic activity, such as the lipopeptides synthesised by *Bacillus* spp. and the rhamnolipids and viscosin produced by certain *Pseudomonas* spp. [29, 30]. Involvement of biosurfactants in microbial adhesion and desorption phenomena has been described previously for streptococci; surface active compounds released by *Streptococcus mitis* not only inhibited the adhesion of *Str. mutans* cells, but also caused *Str. mitis* detachment from glass by modifying the substratum surface [31]. Similarly, a *Str. thermophilus* strain produced a biosurfactant causing its own desorption from glass and leaving a completely anti-adhesive coating [32].

Adsorbed surlactin layers were particularly effective at preventing adhesion to silicone rubber of *Ent. faecalis, E. coli* and *S. epidermidis* strains, but less effective against *K. pneumoniae, Prov. stuartii*, and *P. aeruginosa*. This might be related to the latter, like *Pr. mirabilis*, frequently being urease-positive [33]. Urease converts urea to ammonia, thus enabling bacteria to utilise urea as a source of nitrogen and facilitating rapid growth in urine. Rapid, surface-associated bacterial growth in urine might therefore explain why surlactin layers could not effectively reduce the initial adhesion of certain of these strains. Confirmation of this hypothesis would require the measurement

of urease levels in the flow chamber, which was beyond the scope of the present study.

Unfortunately, most studies on the interference of lactobacilli in uropathogen adhesion or biofilm formation have been carried out in buffer or medium, but rarely in human urine. Besides components affecting bacterial growth, urine also contains a variety of glycoproteins and free oligosaccharides that are analogous to the cell-bound receptors for bacterial adhesins. These soluble receptor analogues can interact with micro-organisms and in this way influence their adhesion to the uroepithelium or biomaterial surfaces. It has been demonstrated, for example, that Tamm Horsfall protein (THP), present at the renal epithelial surface and the most abundant glycoprotein found in normal human urine, inhibits the adhesion of type 1 fimbriate *E. coli* to uroepithelial cells by binding *E. coli* [34]. THP and human urine were also found to alter the adhesion profiles of uropathogenic type 1 fimbriate *E. coli, P. aeruginosa, S. epidermidis* and *Pr. mirabilis* to various polymer substrata with respect to phosphate-buffered saline, but no single trend was apparent [35].

Although the *L. acidophilus* RC14 biosurfactant surlactin did not cause uniform inhibition of initial adhesion of all the uropathogens tested, it was effective against the majority and this demonstrates that surlactin shows promise for the development of an anti-adhesive biological coating for catheter materials.

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