# A Surface-Active Agent from *Saccharomyces cerevisiae* Influences Staphylococcal Adhesion and Biofilm Development

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Bacterial biofilms which are responsible for a number of diseases are very difficult to control effectively because of their high resistance to antibiotics and the host defence system. The use of natural products decreasing or preventing initial adhesion of bacteria and biofilm formation is one of the alternative therapeutic strategies taken into consideration. We ask the question, whether a crude extract from the cell wall of *Saccharomyces cerevisiae* (mannoprotein), which possesses surfactant activity, may be used as inhibitor of *Staphylococcus aureus* and *S. epidermidis* biofilm development. By using the "bactericidal spot assay" it was demonstrated that mannoprotein had no direct antibiotic activity against the tested strains. The influence of this extract on initial adhesion, biofilm formation and dispersal of preformed biofilms was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. In this assay, live bacteria with an active electron transport system reduce the tetrazolium salt to a water-soluble purple formazan product, and optical density reading ($A_{550}$) values are directly dependent on their cell numbers. Yeast-derived surfactant, when adsorbed in the microplate wells or present in the medium, was effective both in decreasing the initial deposition of staphylococci and in reducing the amount of growing biofilm, quantitated after 24 h of co-incubation with the bacteria. It also changed the parameters of biofilm morphology analyzed by PHLIP – the confocal laser scanning microscopy image quantification package. Mannoprotein also accelerated the detachment of mature staphylococcal biofilms, preformed in optimal conditions. It was concluded that mannoprotein anti-biofilm action reflects its influence on cell surface hydrophobicity.

*Key words:* Mannoprotein, Staphylococci, Biofilm

## Introduction

The communities of microorganisms called biofilms are involved in the pathogenesis of various infections, and staphylococci are an example of bacteria which are very potent biofilm makers (Fux *et al*., 2005; Goetz, 2002). Such biofilm-associated infections, caused by *Staphylococcus aureus* or *S. epidermidis* and often connected to the use of medical devices, are very difficult to treat effectively because of their high resistance to antibiotics and the host defence system. Many research groups have investigated potential strategies to prevent biofilm formation or to eradicate biofilms, which could be accessory or alternative to antibiotics (Lewis, 2001; Fux *et al*., 2005). One possible approach, which seems to be a very attractive idea, is prevention of biofilm formation by surface conditioning or biofilm disruption/removal by natural products, such as enzymes or surface-active agents (surfactants) derived from other microorganisms (Shintani, 2004; Singh and Cameotra, 2004; Walencka *et al*., 2005; Rodrigues *et al*., 2006). A variety of surface-active agents produced by bacteria and fungi has different chemical structures and natural roles for the life of producers. They can be released from the cell, bound to their surface or are integral cell wall components (Bernhard *et al*., 2000; Rodrigues *et al*., 2006). Several surfactants are known to have direct antimicrobial properties playing a role in regulating the competitive microorganisms' attachment-detachment to and from surfaces. Biosurfactants are also involved in bacterial pathogenesis, quorum sensing-dependent communication and biofilm formation (Ron and Rosenberg, 2001). However, there are many other

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surfactants whose physiological function is not known yet. The majority of surface-active components have been reported in bacteria but recently filamentous fungi and yeasts have been used extensively for the production of surfactants, because of low production costs and no hazard on human health when used in food, cosmetic or pharmaceutical products (Lukondeh *et al*., 2003; Rodrigues *et al*., 2006). Taken this information into account, in the present study we ask the question whether yeast-derived biosurfactant, chemically known as mannoprotein, may be considered as an effective agent against biofilms formed by *S. aureus* and *S. epidermidis* strains.

## Materials and Methods

### *Bacteria*

*Staphylococcus aureus* (reference ATCC 29213; 2 clinical isolates: A3, 1474/01) and *S. epidermidis* (typical slime-producing RP12; 2 clinical isolates: A4c, 6756/99) strains were stored in TSB (tryptic soy broth; Difco) with 15% glycerol at −70 °C.

### *Surfactant extraction and antibacterial activity testing*

Cell wall extract of *Saccharomyces cerevisiae* yeast (wine Tokay strain) was obtained using the method of Cameron *et al*. (1988) slightly modified by Torabizadeh *et al.* (1996). Yeasts were grown in liquid medium [0.5% (w/v) yeast extract, 1% peptone, 1% glucose] for 2 d at 28 °C with shaking. Yeast culture was centrifuged, washed twice in distilled water and suspended at 20% (wet weight; by volume) in 0.1 M potassium citrate/0.02 M potassium metabisulfite (adjusted pH = 7.0). This mixture was autoclaved for 2 h (121 °C) and then centrifuged at $5{,}000 \times g$ for 10 min at room temperature. For preparation of biosurfactant, 3 volumes of 95% ethanol containing 1% (v/v) acetic acid were added to the supernatant for 16 h at 4 °C. The precipitate was collected by centrifugation ($10{,}000 \times g$), dried and stored at room temperature. Surfactant activity of the obtained extract was evaluated as its emulsification activity according to the method of Akit *et al.* (1981) and Cameron *et al.* (1988). Extract (0.1 g) was dissolved in 4 ml of distilled water, and 6 ml of kerosene oil were added, then vortexed to homogeneity and left at room temperature. After 1 h the proportion of kerosene oil emulsified was compared with the total volume of kerosene oil added and the percentage of emulsion was calculated. Water (4 ml) vigorously mixed with kerosene oil (6 ml) was used as the control.

Antibacterial activity of mannoprotein (100 mg/ml in PBS) was assessed by spotting 25 $\mu$l onto Mueller-Hinton agar plates (Difco) previously seeded with 1 ml of $10^8$ CFU of fresh overnight cultures of *S. aureus* or *S. epidermidis* strains. Spotted plates were incubated at 37 °C for 24 h before the evaluation of growth inhibition zones.

### *Cell surface hydrophobicity*

A salt aggregation test (SAT) was used to determine the bacterial cell surface hydrophobicity (CSH). This was performed by mixing 100 $\mu$l of freshly prepared standard bacterial suspension [approx. $5 \times 10^8$ bacteria non-treated or treated with mannoprotein (100 mg/ml in PBS) for 24 h at 37 °C] with 100 $\mu$l of ammonium sulfate solution (0.2–4.0 M in PBS, pH 6.8) in a 24-well culture plate (Nunclon®, Nunc, Roskilde, Denmark). The lowest salt concentration at which visible aggregation of bacteria occurred was taken as a measure of the SAT value using a phase-contrast microscope (magnification ×200, Nikon Eclipse TE 2000-S; Nikon, Kanagawa, Japan).

### *Staphylococcal adherence and biofilm formation*

Bacteria were grown for 24 h at 37 °C in 5 ml of TSB supplemented with 0.25% D-(+)-glucose (TSBGlc). The cultures were diluted 1:40 in TSBGlc and 200 $\mu$l were added to the wells of a 96-well tissue culture-treated polystyrene plate (Nunclon™ Surface No. 167008; Nunc). The adherence or biofilm formation in the absence or presence of mannoprotein (concentration range 3.1–100.0 mg/ml in PBS) was evaluated after, respectively, 3 h or 24 h incubation at 37 °C. The influence of mannoprotein (100 mg/ml) on the mature (one-day-old) biofilm was also tested by its treatment with surfactant for 24 h. After the above mentioned treatments biofilm mass was stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, USA] as described earlier (Walencka *et al*., 2005). In this assay, live bacteria with an active electron transport system reduce the tetrazolium salt to a water-soluble purple formazan product and optical density reading ($A_{550}$) values are directly dependent on their cell numbers. Briefly, the wells containing initially adhered cells or formed biofilms were

emptied and stained with MTT solution (0.3% in PBS) for 2 h at 37 °C. MTT was replaced by 150 $\mu$l of DMSO (dimethyl sulfoxide) and 25 $\mu$l of glycine buffer (0.1 M, pH 10.2) for 15 min at room temperature. The colour intensity of the soluble formazan was determined using a microplate reader (Victor2 multifunctional counter, Wallac, Finland) at 550 nm wavelength.

*Confocal microscopy study*

Fresh bacterial cultures diluted 1:40 in TSBGlc were added (500 $\mu$l) to the chambers of Lab Tek chamber slide II (Nalge-Nunc Int., Rochester, USA) for 24 h at 37 °C. Three series of experiments were carried out in order: (i) to compare the biofilm formation in chambers not treated or previously conditioned with mannoprotein; (ii) to compare the biofilm formation in the absence or presence of mannoprotein in the culture medium; (iii) to evaluate the mannoprotein activity against one-day-old biofilms preformed under optimal conditions. Biofilms grown on the slides surface not treated or treated with biosurfactant (100 mg/ml) as described above were stained with 0.1% FITC (fluorescein isothiocyanate; Sigma) solution in PBS for 20 min at room temperature. Examination of FITC-stained biofilms was performed using LSM5 (Pascal) confocal laser scanning microscopy (CLSM) with an Axiovert 2 (Zeiss) microscope possessing a Plan-Apochromat 100× (1.4 oil) objective. Images were recorded at a 488 nm excitation and emission at 530 nm (long pass filter set). Digital image analysis of CLSM optical thin sections was performed with Pascal Zeiss software. Values for the image analysis parameters were calculated by the PHLIP quantification package as described by Mueller *et al.* (2006).

*Statistical analysis*

Differences in biofilm structure measured with PHLIP were tested for significance using Student's test. Experiments were conducted in triplicate, and at least 10 fields per each sample were collected. Results are presented as the mean ± SD.

## Results and Discussion

*Biosurfactant preparation and characterization*

Ethanol/acetic acid precipitation of autoclaved *S. cerevisiae* cell suspension allowed the isolation of extract yielding 12% of the yeast cell weight. Its kerosene oil emulsification activity was estimated as 80%. Data of the agar spot test revealed no direct antibiotic activity of the extract against *S. aureus* and *S. epidermidis* strains. It is well known that the yeast-derived biosurfactants are mainly glycolipids (mannosylerthritol lipids) or glycoproteins (liposan, mannoprotein). They may (mannosylerthritol lipids) or may not (mannoprotein) have antimicrobial activity (Ron and Rosenberg, 2001). One of the two known classes of mannoproteins extracted form the yeast cells are structural mannoproteins which are interspersed within a network of glucan to form the outer layer in the yeast cell wall. The second one are the periplasmic mannan enzymes (Cameron *et al.*, 1988; Barriga *et al.*, 1999; Lukondeh *et al.*, 2003; Singh and Cameotra, 2004; Rodrigues *et al.*, 2006).

According to the data published earlier (Cameron *et al.*, 1988; Torabizadeh *et al.*, 1996), the extract obtained in this way contains mannoprotein composed of approximately 90% carbohydrate (mannan) and 4–10% protein which is responsible for its action as emulsifier. Based on these data, in the present study a crude preparation was used, without further purification. However, from the high yield of our product and its effective emulsification, it can be concluded that it consists mostly of structural mannoproteins, as the mannan enzymes do not co-sediment with the cell wall fragments after the breakage of the cells (Cameron *et al.*, 1988). The protein content in our crude extract, estimated using the BioRad assay with bovine serum albumin (BSA, 10–1000 $\mu$g/ml) as standard, was 7.2%, which is lower than that of the mannan enzymes. The protein part of our extract gave in SDS-PAGE (15% gel) several bands with molecular weights in the range 15–25 kDa, visible despite the carbohydrate smearing. The similar observations were made by Torabizadeh *et al.* (1996).

*Influence of mannoprotein on cell surface hydrophobicity*

To evaluate the ability of biosurfactant to influence the cell wall hydrophobins expression, cell surface hydrophobicity (CSH) was tested by determining the rate of aggregation in ammonium sulfate solution (SAT, salt aggregation test). *S. aureus* strains initially expressed a high or moderate CSH (SAT values: 0.1–0.5 or 0.5–1.5 M, respectively), whereas cells of *S. epidermidis* strains were more

hydrophilic (SAT > 1.5 M). The decrease in *S. aureus* CSH (SAT > 2.0 M) was observed after the co-incubaction of bacteria with mannoprotein for 24 h at 37 °C. On the contrary, CSH of *S. epidermidis* strains treated with mannoprotein was not that significantly changed (data not shown).

*Inhibition of initial adhesion of staphylococci by mannoprotein*

The results obtained using the MTT reduction assay performed in 96-well microplates showed that mannoprotein present in the culture medium was effective in decreasing, in a dose-dependent manner, the initial deposition rates of *S. aureus* and *S. epidermidis* estimated after 3 h of co-incubation. The most effective concentration of biosurfactant which reduced bacterial adhesion by 66–86% was 100 mg/ml (data not shown). A lower concentration range of mannoprotein was effective as anti-adhesion agent against *S. aureus* strains (100–12.5 mg/ml) rather than against *S. epidermidis* strains (100–50 mg/ml). The number of bacterial cells adhering to the wells with preadsorbed mannoprotein (100 mg/ml, overnight) was also decreased, almost to the same degree as it was observed for mannoprotein activity, when this agent was permanently present at the same concentration in the culture medium (data not shown). It can be suggested that mannoprotein, like other surface-active agents, changes the surface properties making it less supportive for bacterial deposition. It is also possible that, by decreasing bacterial cell surface-exposed hydrophobins, this biosurfactant negatively influences the adhesion abilities of microorganisms. It is known that bacterial adhesion may involve, apart from adhesin-receptor *in vivo* interactions, different factors such as hydrophobic and other non-specific interactions under different environmental conditions (Ofek *et al.* 2003; Doyle, 2000).

*Inhibition of biofilm formation*

The preparation of *S. aureus* and *S. epidermidis* biofilms in the wells of a microplate produced high bacterial yield. After MTT staining, colour intensity readings ($OD_{550}$) for all strains ranged from 2.6 to 3.1, therefore these strains were recognized as strong biofilm producers. To analyze the effect of mannoprotein on biofilm formation, either the wells were coated with surfactant (100 mg/ml) or surfactant was added to the growth medium (3.1–

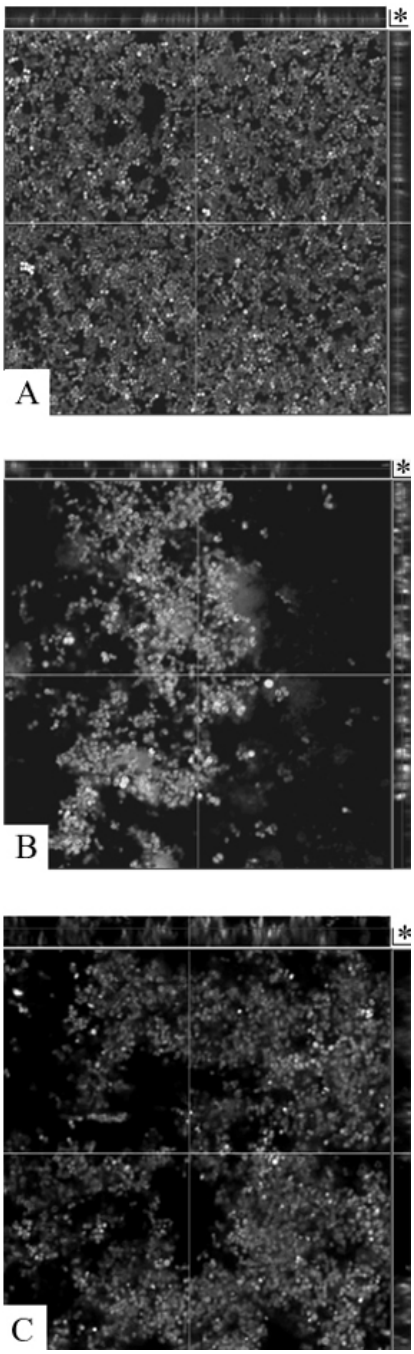


Fig. 1. Confocal laser scanning microscopy images of *S. aureus* ATCC 29213 biofilm formed in chamber slides (24 h at 37 °C): (A) in culture medium; (B) in culture medium + mannoprotein; (C) in culture medium but on slides with preadsorbed mannoprotein. The square panels are a plain view and the side panels are vertical cross sections of the biofilm stained with FITC, as described in Materials and Methods. ⌞* = 4 $\mu$m.

100 mg/ml). When mannoprotein was present in the culture medium, the MTT reduction assay revealed that the effective inhibition of biofilm growth of *S. aureus* and *S. epidermidis* could be achieved. However, such an effect was strain- and dose-dependent: the most significant decrease in biofilm mass (12–87%) was observed when mannoprotein was used in a concentration of 100 mg/ml. Mannoprotein also slightly decreased the biofilm formation by *S. aureus* and *S. epidermidis* strains when polystyrene wells were coated with this agent before inoculation of bacteria (data not shown).

To test if mannoprotein would dislodge a preformed biofilm, it was added to the wells after the bacterial culture reached confluent mass (24 h). Dispersal of biofilm clumps caused by biofilm incubation with mannoprotein was observed in the case of all tested staphylococcal strains, however, the intensity of the effect was more efficient for *S. epidermidis* (31–82%) versus 10–42% of biomass reduction for *S. aureus* strains (data not shown).

Such anti-biofilm activity of mannoprotein was confirmed in a separate set of experiments using laser scanning confocal microscopy (CLSM). Microscope images showed multilayered clumps of bacteria, surrounded by less dense material, loosely attached to the surface. It means that the biofilm structure was disrupted by mannoprotein treatment (Figs. 1A, B, C). The quantitative description of biofilm morphology performed by PHLIP analysis enabled the testing for a change of particular quantifiers in response to mannoprotein treatment and depending on the time of culture. Variations in the morphological parameters occurred over time and were strain-dependent. But generally, when mannoprotein was introduced to the culture medium along with bacterial inoculum for 24 h, it resulted in staphylococcal biofilms covering less (2- to 4-times) surface area, decrease (20–50%) in their total biovolume and lower mean biofilm thickness. Also morphological parameters, like horizontal and vertical spread of biomass, were negatively affected by the mannoprotein presence in comparison to the non-treated control biofilms. The examples of PHLIP analysis of the surfactant's influence on *S. aureus* and *S. epidermidis* biofilm development are presented in Table I and Table II, respectively. When mannoprotein (100 mg/ml) was added to the biofilms preformed in optimal conditions, their effect was also noticeable after further 24 h incubation and PHLIP analysis also showed that all measured parameters were negatively affected (data not shown).

Table I. The influence of mannoprotein on biofilm formation by *S. aureus* strains (CLSM images PHLIP analysis).

| Parameters of biofilm morphology | *S. aureus* ATCC 29213 | | *S. aureus* A3 | | *S. aureus* 1474/01 | |
|---|---|---|---|---|---|---|
| | Control | MP[a] | Control | MP[a] | Control | MP[a] |
| Total biovolume [$\mu m^3$] | 9671.82 ± 345.33 | 2817.10[b] ± 2097.87 | 25577.92 ± 7206.12 | 10267.95[c] ± 2754.06 | 17956.75 ± 2409.17 | 11347.06[b] ± 1091.03 |
| Substrate coverage (%) | 55.35 ± 7.68 | 19.90[c] ± 5.75 | 69.74 ± 10.18 | 19.08[c] ± 2.85 | 72.67 ± 6.12 | 40.17[c] ± 5.63 |

[a] *S. aureus* biofilms were grown for 24 h at 37 °C in the presence of mannoprotein (MP) at the concentration 100.0 mg/ml.
[b] $P < 0.05$.
[c] $P < 0.01$.

| Parameters of biofilm morphology | *S. epidermidis* RP12 | |
|---|---|---|
| | Control | MP[a] |
| Total biovolume [$\mu m^3$] | 37315.30 ± 11475.89 | 4794.10[c] ± 1499.44 |
| Substrate coverage (%) | 57.10 ± 13.53 | 16.99[c] ± 2.05 |
| Mean thickness [$\mu m$] | 6.89 ± 1.07 | 3.28[c] ± 0.33 |
| Horizontal spreading [$\mu m^2$] | 1336.44 ± 90.75 | 762.31[c] ± 162.16 |
| Vertical spreading [$\mu m^2$] | 6.44 ± 2.14 | 0.96[b] ± 0.23 |

Table II. CLSM images PHLIP analysis of *S. epidermidis* RP12 biofilm structure.

[a] *S. epidermidis* RP12 biofilms were grown for 24 h at 37 °C in the presence of mannoprotein (MP) at the concentration 100.0 mg/ml.
[b] $P < 0.05$.
[c] $P < 0.01$.

The way in which various biosurfactants influence bacterial adhesion and biofilm construction seems to be connected with surface tension changing and bacterial cell wall charge changing. These factors are very important in overcoming the initial electrostatic repulsion barrier which exists between the microorganism cell and substratum. Surfactants have the potential to affect both cell-to-cell and cell-to-surface interactions and our results support the opinion that mannoprotein affects these interactions. The ability of surfactants to disrupt bacterial attachment has been observed earlier for the surfactant produced by *Lactobacillus* spp. (surlactin), *B. subtilis* (surfactin, iturin), *P. aeruginosa* (rhamnolipid), *Streptococcus* spp. (rhamnolipid-like), *Candida antarctica* (mannosylerythritol lipids) (Velraeds *et al*., 1998; van Hoogmoed *et al*, 2000; Mireles *et al*., 2001; Davey *et al*., 2003; Espinosa-Urgel *et al*., 2003; Rodrigues *et al*., 2006).

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