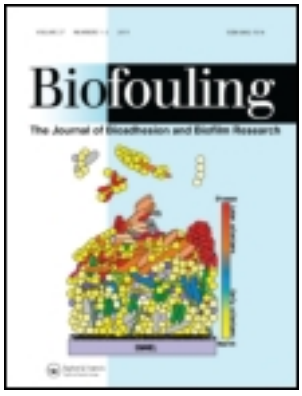

Biofouling
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gbif20

The impact of recombinant fusion-hydrophobin coated surfaces on *E. coli* and natural mixed culture biofilm formation

Annika Rieder [a], Tatjana Ladnorg [b], Christof Wöll [b], Ursula Obst [a], Reinhard Fischer [c] & Thomas Schwartz [a]

[a] Microbiology of Natural and Technical Interfaces Department, Institute of Functional Interfaces, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

[b] Chemistry of Oxidic and Organic Interfaces Department, Institute of Functional Interfaces, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

[c] Microbiology Department, Institute for Applied Biosciences, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany

Available online: 03 Nov 2011

To cite this article: Annika Rieder, Tatjana Ladnorg, Christof Wöll, Ursula Obst, Reinhard Fischer & Thomas Schwartz (2011): The impact of recombinant fusion-hydrophobin coated surfaces on *E. coli* and natural mixed culture biofilm formation, Biofouling, 27:10, 1073-1085

To link to this article: http://dx.doi.org/10.1080/08927014.2011.631168

Biofouling
Vol. 27, No. 10, November 2011, 1073–1085

Taylor & Francis
Taylor & Francis Group

The impact of recombinant fusion-hydrophobin coated surfaces on *E. coli* and natural mixed culture biofilm formation

Annika Rieder[a], Tatjana Ladnorg[b], Christof Wöll[b], Ursula Obst[a], Reinhard Fischer[c] and Thomas Schwartz[a]*

[a]*Microbiology of Natural and Technical Interfaces Department, Institute of Functional Interfaces, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany;* [b]*Chemistry of Oxidic and Organic Interfaces Department, Institute of Functional Interfaces, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany;* [c]*Microbiology Department, Institute for Applied Biosciences, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany*

(*Received 1 July 2011; final version received 5 October 2011*)

The impact of increased surface hydrophobicity on biofilms regarding retardation, repulsion, or attraction was studied with hydrophobin modified glass substrata. Recombinantly produced fungal hydrophobins forming self-assembled monolayers were used as the surface coating. The adsorption dynamics of hydrophobins were analysed with a quartz crystal microbalance which showed the surface coating to be rapid and stable. The change of surface wettability was determined by water contact angle measurements and demonstrated an increase in hydrophobicity in range of 60–62°. The homogeneity of the monolayers was demonstrated by immunofluorescence microscopy. Atomic force microscopy was applied to visualise the uniform texture of the coated materials. The hydrophobin coatings had no impact on different biofilms in terms of spatial distribution, cell numbers, and population composition. In consequence, hydrophobicity might not represent an important parameter for biofilm formation. Nevertheless, recombinant hydrophobins are suitable for large scale surface modification and functionalization with bioactive molecules.

Keywords: recombinant fungal hydrophobins; surface modification; wettability; biofilm formation

Introduction

Microbial biofilms represent a very successful bacterial life form in which the microorganisms benefit from metabolic exchange, genetic flexibility, and protection. Bacterial biofilm communities exhibit properties, behaviours, and survival strategies that by far exceed their capabilities as individual bacteria (Costerton et al. 1999, 2002). Bacteria can establish biofilms as a kind of permanent growth form on a wide variety of natural and synthetic surfaces. Due to this, biofilms represent a major problem especially in environmental biology, medical fields, the food industry, and biotechnology. After a few weeks, compartments exposed to non-sterile matrices regularly exhibit persistent colonization with various bacterial species (Boe-Hansen et al. 2003).

A promising approach to retard, repulse or attract biofilm formation is surface modification. The surface properties of a material, eg its mechanical properties, structure, polarity or chemical properties, influence the adhesion of various molecules and cells (Rosenhahn et al. 2008). The bacterial adhesion process includes an initial physico-chemical interaction phase in which surface wettability is believed to play an important role (Dexter et al. 1975; Pringle and Fletcher 1983; Wiencek and Fletcher 1997). However, in addition to the hydrophobicity, surface roughness and protein adsorption are also recognized as essential for bacteria to colonize a surface (Donlan 2002; Palmer et al. 2007).

With surface modification the biocompatibility and/or biofunctionality of a material can be changed. Various approaches, which change the surface characteristics, have been published but often toxic compounds are used. The use of natural, non-toxic surface-active substances, like hydrophobins, makes surface coatings less hazardous to the environment and represents an alternative to toxic compounds.

Hydrophobins are fungal proteins which are among the most surface-active substances. They are capable of self-assembling into amphiphilic films at hydrophilic–hydrophobic interfaces (Wösten and De Vocht 2000) and, thus, change surface wettability. Hydrophobins are 70–120 amino acids long and have a molecular mass of approximately 10 kDa. They have eight conserved cysteine residues which form four intramolecular disulphide bridges (Wessels 1997). These keep the hydrophobins in a soluble monomeric state and allow self-assembly only if the molecules encounter a hydrophilic–hydrophobic interface (De Vocht et al. 2000). Class I hydrophobins assemble into highly stable films

*Corresponding author. Email: thomas.schwartz@kit.edu

ISSN 0892-7014 print/ISSN 1029-2454 online

http://dx.doi.org/10.1080/08927014.2011.631168
http://www.tandfonline.com

that can only be dissolved in concentrated trifluoroacetic or formic acids (De Vries et al. 1993; Wösten et al. 1993). Due to their properties, numerous applications have been proposed for hydrophobins, including use as biomaterials and in medical and antifouling applications (Hektor and Scholtmeijer 2005).

The aim of this study was to characterise two recombinant fusion-hydrophobins, H*Protein A and H*Protein B, in terms of surface modification. Moreover, the impact of hydrophobin coated surfaces and the resulting change in surface hydrophobicity on microbial biofilm formation was also evaluated.

First the adsorption behaviour of the fusion-hydrophobins was analysed with quartz crystal microbalance measurements (QCM-D) and a coating protocol was developed. The influence of temperature and time on the self-assembly of the recombinant hydrophobins and the change in surface hydrophobicity was determined using water contact angle measurements (WCA). Immunofluorescence microscopy and atomic force microscopy (AFM) were applied to analyse the homogeneity and the texture of the hydrophobin coating and its properties on the nanoscale.

The influence of H*Protein A and H*Protein B coatings and changed surface wettability on adhesion and biofilm formation by *Escherichia coli* and natural wastewater populations was characterised with conventional plating experiments to determine the number of bacteria adhering to the surfaces and fluorescence microscopy to analyse the spatial distribution of the biofilms. Molecular-biological methods, such as denaturing gradient gel electrophoresis (DGGE), were used to determine differences in the composition of the microbial population on the surfaces.

Materials and methods

Preparation of hydrophobin solutions

The recombinant fusion-hydrophobins H*Protein A and H*Protein B were obtained from BASF-SE, Ludwigshafen, Germany. They consist of the class I hydrophobin DewA of *Aspergillus nidulans*, the N-terminal protein yaaD or a truncated form of yaaD of *Bacillus subtilis*, and a C-terminal His-tag (Wohlleben et al. 2010). H*Protein A has a molecular weight of 46.5 kDa and H*Protein B of 19.1 kDa.

Hydrophobin solutions (10 μM) were prepared freshly for each experiment. The proteins were dissolved in coating buffer (50 mM Tris, 1 mM $CaCl_2$, pH 8.0) by stirring for 1–2 h at room temperature. The protein concentration of the hydrophobin solutions was determined before and after use for surface coating using the bicinchoninic acid (BCA) assay (Smith et al. 1985). No significant concentration changes due to surface coating were detected.

Surface coating

Coating was accomplished according to Janssen et al. (2004). Glass slides (soda-lime-silica glass; Menzel-Gläser, Braunschweig, Germany) were cleaned with 60% ethanol, incubated in hydrophobin solution for 1, 6, or 16 h at 20°C or 80°C and subsequently washed three times for 10 min with distilled water and air-dried at ambient temperature. The surfaces were further treated at 80°C in 2% SDS solution for 10 min to test the stability of the coating and to induce the stable β-sheet end conformation. Finally, the modified surfaces were washed and dried as described above.

In addition, glass surfaces were modified with bovine serum albumin (BSA; Sigma-Aldrich, Muenchen, Germany) according to the protocol described.

Surface characterization

WCA measurements

The surface hydrophobicity of glass surfaces before and after hydrophobin coating was measured at ambient temperature with an optical contact angle meter (DSA100, Kruess, Hamburg, Germany) using the sessile drop measuring method (van der Mei et al. 1991). To determine the static contact angles of 5 μl water droplets the drop shapes were evaluated using the Young-Laplace Equation (software DSA3, Kruess, Hamburg, Germany). The WCA values were averaged from five independent measurements on two separately coated surfaces.

Immunofluorescence staining

The His-tag of the recombinant fusion-hydrophobins was used for specific immunofluorescence labelling of the hydrophobin coating.

All incubation steps were carried out at room temperature. First, the hydrophobin-coated and bare glass slides were incubated for 30 min in phosphate buffer solution (PBS) containing 0.1% Tween 20 and 5% milk powder and subsequently for 1 h in primary antibody solution (diluted 1:3000 in PBS containing 0.1% Tween 20 and 1% milk powder; Anti-His tag, Clone His.H8, Millipore, Schwalbach, Germany). After washing the slides thoroughly in PBS containing 0.1% Tween 20 four times for 5 min, the Cy3.5-conjugated secondary antibody (diluted 1:5000 in PBS containing 0.1% Tween 20; goat polyclonal secondary AB to mouse IgG (Cy3.5), abcam, Cambridge, UK) was applied. The slides were incubated for 1 h in the dark, washed as described above, and air-dried. Subsequently, the anti-fading solution citifluor (Citifluor Ltd, London, UK) was applied and the samples were analysed with 630-fold magnification using the Axioplan 2 imaging system (Carl Zeiss, Oberkochen,

Germany) with the filter set BP 546/12, FT 580, LP 590. Ten digital images were obtained of each sample with a Zeiss AxioCam MRm camera and the Axio Vision 4.6 software.

Atomic force microscopy (AFM)

AFM measurements were performed on bare and hydrophobin-modified glass slides deposited on the scanner of an MFP-3D BioAFM (Asylum, Mannheim, Germany) having a functionalised (hydrophobic/hydrophilic) Si_3N_4 cantilever with a normal spring constant of 0.6 N m^{-1} (μmash) in air. The microscope was operated in the AC mode, where the tip was scanned back and forth at 0° along the horizontal line in a scan range of 5 μm and 1 μm, respectively. The AFM was used in a closed loop on all three axes. Five topographic images of each sample were evaluated using the corresponding IGOR software.

Quartz crystal microbalance with dissipation monitoring (QCM-D)

A Q-Sense E4 instrument (LOT-Oriel, Darmstadt, Germany) with four parallel sample chambers was used to analyse the adsorption behaviour of H*Protein A and H*Protein B on SiO_2-coated quartz crystals (QSX 303, LOT-Oriel, Darmstadt, Germany). The variation in frequency (Δf) and dissipation (ΔD) were monitored as a function of time during the measurements using the software QSoft 401 (Version 2.5.8.527) supplied by Q-Sense. The quartz crystals were cleaned right before use in a UV-ozone chamber (UV/Ozone ProCleaner, BioForce Nanosciences, Ames, USA) for 20 min. All experiments were carried out at a constant flow rate of 50 μl min^{-1} at 20°C, and with a baseline corresponding to the loading of the crystal with coating buffer (50 mM Tris, 1 mM $CaCl_2$, pH 8.0). The hydrophobin adsorption was monitored for up to 16 h before the crystals were rinsed with coating buffer. At least three repetitions were performed for each experimental setup.

For data evaluation, the QTools software (Version 3.0.11.512) was used. From QCM-D frequency and dissipation data obtained at the relevant overtones, the deposited layer thickness and mass were calculated using Voigt viscoelastic modelling.

Biofilm cultivation

Biofilms on hydrophobin-coated and uncoated glass slides were cultivated in a biofilm flow through reactor (in-house construction: length 29.0 cm, inner diameter 4.6 cm) at a constant flow rate of 10 ml min^{-1} at 20°C.

The reference strain *E. coli* (*E. coli* BW3110, pJOE 4056.2 His e-GFP) used in this study had a rhamnose inducible promotor for intracellular expression of the green fluorescent protein (GFP) (Wilms et al. 1999; Wegerer et al. 2008). Overnight cultures of single bacterial colonies were diluted with 1:4 Brain heart infusion broth (BHI broth) (Merck Eurolab, Darmstadt, Germany) containing 0.2% rhamnose and incubated at 37°C until the bacterial cells reached the exponential phase (OD_{600nm} 0.5–0.7). This suspension was diluted with 1:4 BHI containing 0.2% rhamnose to an OD_{600nm} 0.1 and used to start biofilm cultivation. As nutrient solution, 1:4 BHI containing 0.2% rhamnose was used. After 5, 17, and 26 h, respectively, the glass slides were removed from the reactor and washed in sterile cell wash buffer (5 mM Mg acetate, 10 mM Tris, pH 8.0).

For natural multispecies biofilms, wastewater from a municipal sewage plant was used as inoculum. OECD synthetic sewage (ISO International Standards Organisation, Method 11733, 2004) was used as nutrient solution. It was prepared from a l00-fold concentrated solution consisting of 16 g peptone, 11 g meat extract, 3 g urea, 0.7 g NaCl, 0.4 g $CaCl_2 \times 2 H_2O$, 0.2 g $MgSO_4 \times 7H_2O$, and 2.8 g K_2HPO_4, anhydrous) in 1000 ml of distilled water (pH 7.5 $\pm$ 0.5). After an incubation time of 4 weeks, the glass slides were removed from the reactor and washed in fresh synthetic sewage.

Biofilm characterization

Fluorescence microscopy

The adhesion of *E. coli* to bare and hydrophobin-modified glass slides was analysed with 100 to 1000-fold magnification using the Axioplan 2 imaging system (Carl Zeiss, Oberkochen, Germany) with the filter set BP 450–490, FT 510, BP 515–565. Digital images of each sample were obtained with a Zeiss AxioCam MRm camera and the AxioVision 4.6 software. The surface coverage of four independent pictures per sample was determined with the BioFlux 200 software (Version 2.3.0.3; Fluxion Biosciences/ IUL Instruments GmbH, Königswinter, Germany).

To characterise the spatial distribution of the natural mature biofilms, the bacteria were stained with the dye Syto9 (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol and subsequently analysed as described above.

Colony-forming units (CFU)

Each glass slide was thoroughly scraped with a sterile cell scraper and a homogeneous bacterial suspension was prepared in phosphate buffer solution (PBS). The number of cultivable adherent bacteria was quantified by means of the pour-plate method, ie plating serial dilutions of bacterial suspensions obtained on the

triple scale. R2A-agar (Merck Eurolab, Darmstadt, Germany) was used as growth medium for heterotrophic bacteria (Reasoner and Geldreich 1985). After incubating the agar plates for 7 days at 20°C, the bacterial colonies were counted and the number of bacteria per square cm was calculated.

Denaturing gradient gel electrophoresis (DGGE)

To analyze the bacterial population growing on hydrophobin-coated and uncoated glass slides and in the planktonic phase, first, the DNA was isolated using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The lysis of the bacterial cells was achieved in Tris/EDTA buffer (20 mM Tris/HCl, 2 mM EDTA, 1.2% Triton, pH 8.0) containing 20 mg ml^{-1} of lysozyme for 2 h at 37°C and subsequent Proteinase K treatment for 45 min at 56°C. The concentration and purity of the isolated DNA were determined with the NanoDrop ND-100 spectral photometer (PeqLab, Erlangen, Germany). The absorbance of DNA was measured at 260 nm and the purity was determined from the absorbance ratio of A260/A280.

Two microlitres of extracted genomic DNA (up to 80 ng) served as a template for the PCR amplification of an internal fragment of the 16S rRNA coding gene using the primers GC27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 517R (5′-ATT ACC GCG GCT GCT GG-3′). The primer GC27F had a GC clamp at its 5′ end (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCG CCC CCG CCC C-3′) (Muyzer et al. 1993; Emtiazi et al. 2003). The final 25 μl PCR reaction mixture contained 1.25 U of Taq-DNA polymerase (peqGold Hot Taq DNA-Polymerase; PeqLab, Erlangen, Germany), 20 pmol of each primer, 10 × PCR buffer, 200 μM dNTPs, water, and template DNA. A GeneAmp PCR System 9700 (Applied Biosystems, Darmstadt, Germany) was used for PCR. The cycling parameters were as follows: initial activation of the polymerase for 30 s at 95°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C followed by a 7 min final elongation step at 72°C. Aliquots of 5 μl were analysed by electrophoresis on a 1% agarose gel containing ethidium bromide to check the sizes and amounts of the amplicons.

DGGE analysis of PCR products was performed with the D-Code-System (Bio-Rad Laboratories GmbH, Munich, Germany) using polyacrylamide gels containing a gradient of 40–70% urea. Eight microlitres of the PCR products were loaded on the gel. DGGE gels were run in 1 × TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.5) at 60°C and 70 V for 16 h and stained with SYBR® Gold (Invitrogen, Karlsruhe, Germany). Finally, the stained gels were analysed at 520 nm wavelength using the LumiImager Working Station (Roche Diagnostics, Mannheim, Germany).

DGGE fingerprints were scored manually by the presence or absence of bands, independently of intensity. The number of bands corresponds roughly to the number of bacterial species in the analysed sample. Community similarities were calculated in a pair-wise manner using the Dice coefficient: $Cs = 2j/(x + y)$ (j = the number of common bands in samples X and Y; x = the total number of DNA bands in sample X; and y = the total number of DNA bands in sample Y). Analysis resulted in Cs values ranging between 0 and 1. The Cs value of 0 reflects no similarity of the two samples, while the Cs value of 1 stands for 100% similarity (Murray et al. 1996).

Results

*Adsorption behaviour of recombinant fusion-hydrophobins H*Protein A and H*Protein B*

The adsorption properties of the recombinant fusion-hydrophobins H*Protein A and H*Protein B were analysed using the quartz crystal microbalance with dissipation monitoring (QCM-D) technique. In addition to the adsorption kinetics, the viscoelastic properties of the adsorbed films and the adsorbed protein amounts were determined.

Figure 1 shows a QCM-D sensogram of adsorption of hydrophobin H*Protein A (10 μM) on a device-specific hydrophilic SiO_2 substratum surface. The x-axis shows the time and the y-axis the change in frequency (Δf) (Figure 1a) or dissipation (ΔD) (Figure 1b) of the measured overtones. Binding of the hydrophobin to the surface is reflected by a decrease in frequency and an increase in dissipation. After injection, H*Protein A adsorbed quickly to the surfaces and the frequency as well as dissipation changes remained constant for 16 h. Similar kinetics were observed for H*Protein B (data not shown). The formed protein layers were stable and practically no bound protein was removed from the surface by a final buffer rinse. H*Protein A (46.5 kDa) caused a change in frequency of up to 80 Hz and a change in dissipation of 6×10^{-6} after buffer rinsing. The adsorption of the smaller recombinant fusion-hydrophobin H*Protein B (19.1 kDa) caused a Δf of up to 65 Hz and a ΔD of 3.5×10^{-6} after the buffer rinse.

Plotting of ΔD as a function of Δf provides information about the rigidity of the layer as a function of the adsorbed amount of protein. The layers formed by the recombinant fusion-hydrophobins had similar viscoelastic properties. The adsorption process was linear and no increase or decrease in rigidity or conformational changes of the hydrophobin layers were detected after initial protein binding. Adsorption

of H*Protein A and H*Protein B induced a change in dissipation of $\sim 1 \times 10^{-6}$ for a change in resonance frequency of 10–12 Hz.

In addition to the qualitative adsorption properties of H*Protein A and H*Protein B, the quantitative adsorption properties were also characterised by the QCM-D technique. Since the relation between the change of the detected QCM-D signals (frequency and dissipation) and the properties of the adsorbed film is not straightforward, a theoretical model was applied to resolve them. For modelling dissipative, viscoelastic protein layers, such as the fusion-hydrophobin layers, the Voigt model can be used to compute the layer thickness and sensed mass (Halthur et al. 2010). Good fits of the data were obtained with parameters for the Voigt model set to a viscosity (kg ms^{-1}) between 0.0005 and 0.01, a shear force (Pa) between 1000 and 1×10^{10}, and a film thickness (m) forced between 1×10^{-11} and 1×10^{-7}. The film density on the SiO_2-coated quartz crystals was assumed to be 1050 kg m^{-3}. H*Protein A formed a 17 ± 1 nm thick layer and the maximum bound level of hydrophobin corresponded to 17 ± 1 mg m^{-2} (Figure 2a). H*Protein B formed a 15 ± 3 nm thick layer and 15 ± 3 mg protein were bound per 1 m^2 (Figure 2b).

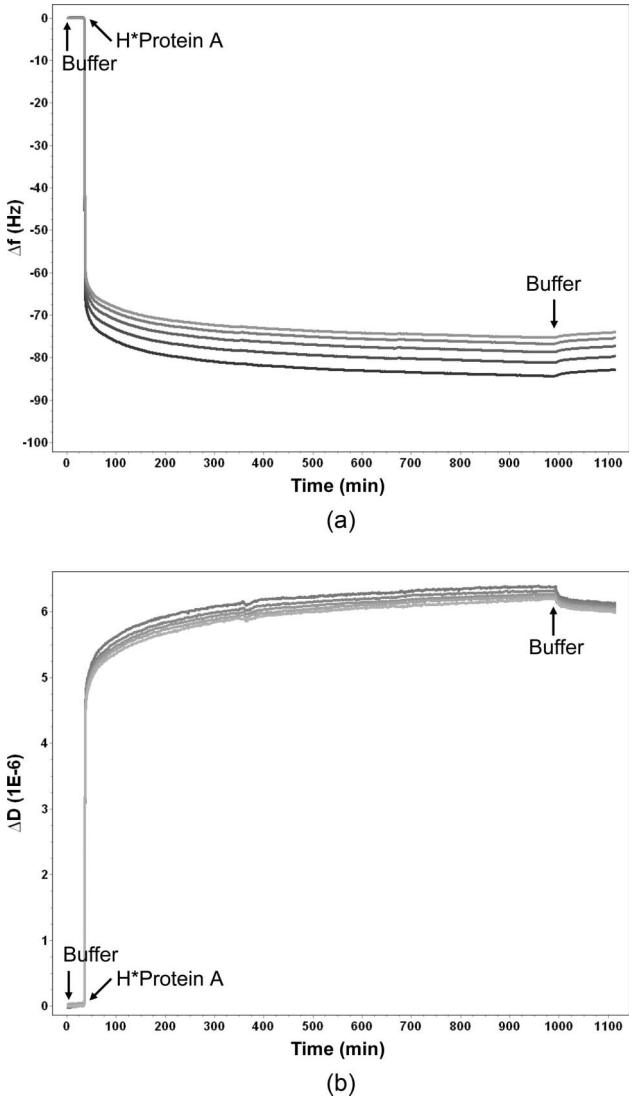

Figure 1. Representative quartz crystal microbalance sensogram of H*Protein A adsorption to device-specific hydrophilic SiO_2 surfaces. Frequency (a) and dissipation (b) change as a function of time during protein adsorption at a concentration of 10 μM, a constant flow rate of 50 ml min^{-1}, and a temperature of 20°C. The data measured for the 5th, 7th, 9th, 11th, and 13th overtone are presented.

Characterisation of hydrophobin-modified surfaces

Glass surfaces were coated with the recombinant fusion-hydrophobins H*Protein A and H*Protein B and characterised with respect to surface hydrophobicity, coating homogeneity and texture.

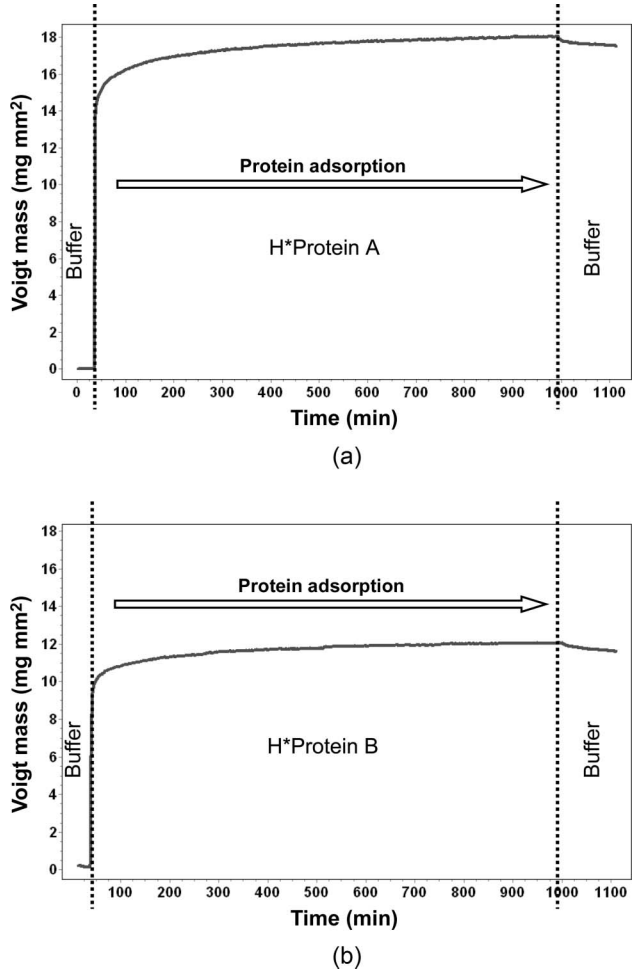

Figure 2. Voigt mass *vs* time during adsorption of H*Protein A (a) and H*Protein B (b) to hydrophilic device-specific SiO_2 surfaces.

Surface hydrophobicity

The ability of the recombinant fusion-hydrophobins H*Protein A and H*Protein B to change surface hydrophobicity was analysed with water contact angle (WCA) measurements. Glass slides were coated with H*Protein A and H*Protein B according to the protocol described. To determine the influence of temperature and time on surface activity and, thus, coating efficiency of the fusion-hydrophobins, temperatures of 20°C and 80°C and incubation times of 1, 6, and 16 h were applied.

Figure 3 shows the contact angles measured on glass slides coated with H*Protein A and H*Protein B. Bare glass is a hydrophilic material with a WCA of 11°. Hydrophobin coating of the glass slides increased surface hydrophobicity. On glass slides coated with H*Protein A (Figure 3a) at 20°C, water contact angles of 56° for 1 h and 57° for 16 h incubation were measured. These experiments demonstrated that the hydrophobins assembled on the glass surfaces within 1 h and longer incubation times did not result in any additional increase in surface hydrophobicity. A temperature effect was obvious when the coating temperature was raised to 80°C. At this temperature, a WCA of 53° was determined on the coated glass surfaces after incubation for 1 h, but at longer incubation times the surface hydrophobicity increased and a contact angle of 73° was reached after 16 h. Surface coatings with H*Protein B showed similar characteristics (Figure 3b). Here, coatings performed at 20°C produced contact angles of 46°and 56° after incubation for 1 h and 16 h, respectively. Longer incubation (16 h) at an elevated temperature (80°C) resulted in an increase in the WCA to 71°. The fusion-hydrophobins H*Protein A and H*Protein B assembled on the glass surfaces and changed the surface hydrophobicity in a temperature- and time-dependent manner.

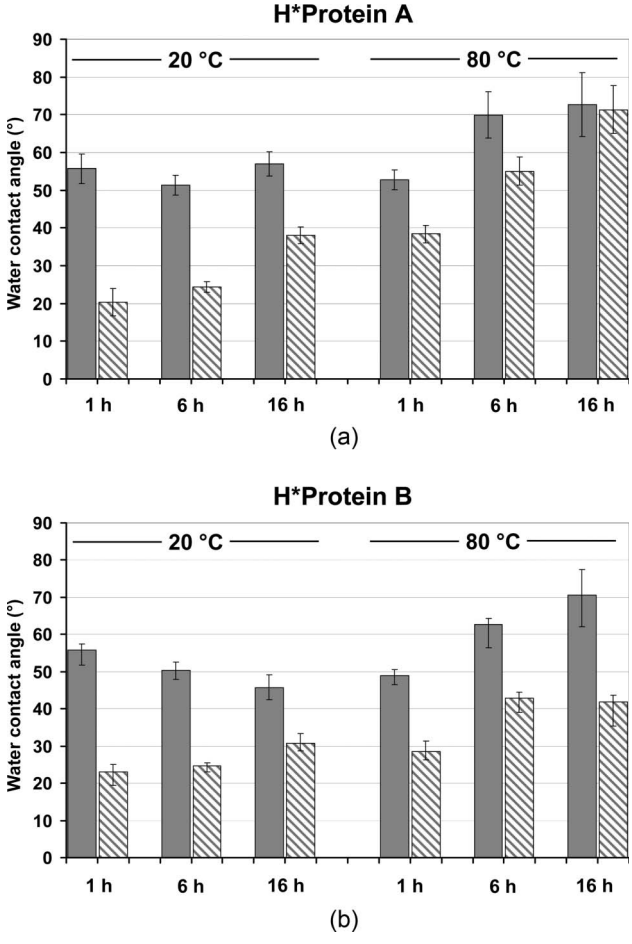

Figure 3. Surface hydrophobicity of glass surfaces coated with H*Protein A (a) and H*Protein B (b) at 20°C or 80°C for 1, 6, or 16 h. Water contact angles were measured before (■) and after (▨) hot SDS treatment. Bare glass surfaces were used as control and had a WCA of 11°. Error bars = the SD of five independent experiments.

The glass surfaces coated with the fusion-hydrophobins were further treated with 2% SDS at 80°C to induce the proposed stable β-sheet end conformation. Parts of the hydrophobin layer and the corresponding surface hydrophobicity were lost after SDS treatment at first. Here, on surfaces coated with H*Protein A (Figure 3a) at 20°C for 1 h and 6 h, contact angles of 20° and 24°, respectively, were measured. On surfaces incubated for 16 h at 20°C, contact angles of 38° were measured indicating a slightly stronger binding to the surface. This effect was intensified at an elevated temperature of 80°C. On surfaces incubated for 1 h and 16 h at 80°C, water contact angles of 38° and 71°, respectively, were measured after SDS treatment. Surface coatings with H*Protein A for 16 h at 80°C were highly stable and not affected by SDS treatment. Surface coatings with H*Protein B showed similar properties (Figure 3b). Here, coatings performed at 20°C for 1 h and 16 h showed contact angles of only 23° and 31°, respectively, after SDS treatment. Coatings with long incubation times (16 h) at an elevated temperature (80°C) were highly stable and still had a contact angle of 42°.

Furthermore, the durability of the hydrophobin coating was analysed. No change in surface hydrophobicity was determined after storage for 4 weeks at room temperature. Glass surfaces coated with H*Protein A for 16 h at 80°C still had water contact angles of 76 $\pm$ 4° before and 74 $\pm$ 5° after SDS treatment. Glass surfaces coated with H*Protein B for 16 h at 80°C had contact angles of 76 $\pm$ 2° and 44 $\pm$ 3° before and after SDS treatment, respectively, after storage for 4 weeks.

Coating homogeneity

Immunofluorescence-labelled antibodies were used to visualise the hydrophobin coating on glass surfaces

with epifluorescence microscopy. Here, the primary antibody bound specifically to the C-terminal His-tag of the recombinant fusion-hydrophobins and a fluorescence-labelled secondary antibody detected and visualised the complexes of primary antibodies and fusion-hydrophobins.

Glass surfaces coated with the recombinant hydrophobins H*Protein A and H*Protein B at 80°C before and after hot SDS treatment were analysed. Bare glass surfaces served as control for immunofluorescence staining. Here, no fluorescence signals were detected.

H*Protein A and H*Protein B assembled on the glass surface at 80°C within 1 h and formed a homogeneous protein layer (Figure 4). Longer incubation (16 h) resulted in a microscopically highly homogeneous coating. The influence of incubation time on coating stability and, hence, homogeneity became apparent after hot SDS treatment. On surfaces incubated for 1 h, major parts of the surface coating were lost due to hot SDS treatment. This loss was much smaller and the coating was more homogeneous on surfaces incubated for a longer time before the SDS treatment. After hot SDS treatment, the hydrophobin coating on surfaces incubated for 16 h was as homogeneous as before SDS treatment.

To analyse the texture of the recombinant fusion-hydrophobin coating on the nanoscale, atomic force microscopy (AFM) was used. Glass surfaces coated for 16 h at 80°C with H*Protein A and H*Protein B were analysed.

H*Protein A formed a homogeneous protein layer on glass surfaces. On the homogeneous protein coating, small protein aggregates were visible. After hot SDS treatment, the glass surfaces coated with H*Protein A (Figure 5) and H*Protein B (data not shown) were still densely packed with proteins. The small aggregates which had adhered to the surface were removed. The films of H*Protein A and H*Protein B were uniform and exhibited a highly ordered structure.

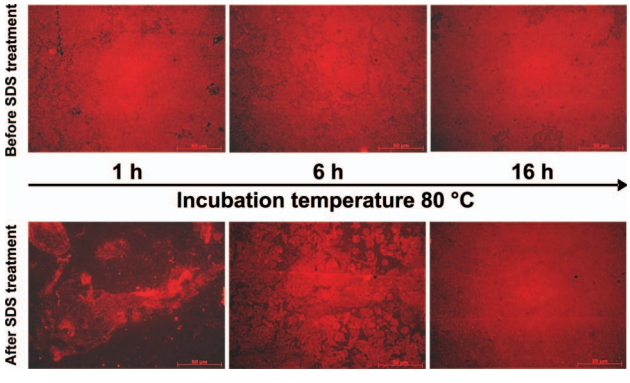

Figure 4. Representative microscope images of immunofluorescence staining of the H*Protein B coating on glass surfaces. The surfaces were incubated in hydrophobin solution for 1, 6, or 16 h at 80°C and analysed before and after hot SDS treatment.

Influence of hydrophobin coatings on initial bacterial adhesion

A characteristic feature of all biofilms is their regulated development in several chronological stages, whereby the initial adhesion of single bacterial cells, the formation of the linking film, is the most critical step.

To monitor bacterial adhesion to bare and hydrophobin-modified glass surfaces, a GFP-tagged *E. coli* (Wilms et al. 1999; Wegerer et al. 2008) served as reference strain. The biofilms were grown in a defined nutrient solution (brain heart infusion, diluted 1:4) in a biofilm flow through reactor for 5, 17, and 26 h, respectively. Prior to fluorescence microscopy, the samples were washed with cell wash buffer to remove loosely attached bacteria, so that only firmly adhered *E. coli* cells were analysed.

Only a few *E. coli* cells had adhered to the surfaces after 5 h. The bacterial cells were unevenly distributed. No cells were detectable in many areas. Bacteria covered 0.15% of the unmodified glass surface after 17 h (Table 1) which corresponded to 3.3×10^3 cells cm^{-2}. The coverage was higher on hydrophobin-modified surfaces with values for H*Protein B coatings of 0.39% before and 0.41% after SDS treatment ($14.5–15.4 \times 10^3$ bacterial cells cm^{-2}). After 26 h, more bacteria had adhered to the H*Protein B-modified surfaces after SDS treatment (surface coverage 0.51%) than to unmodified glass surfaces (surface coverage 0.26%).

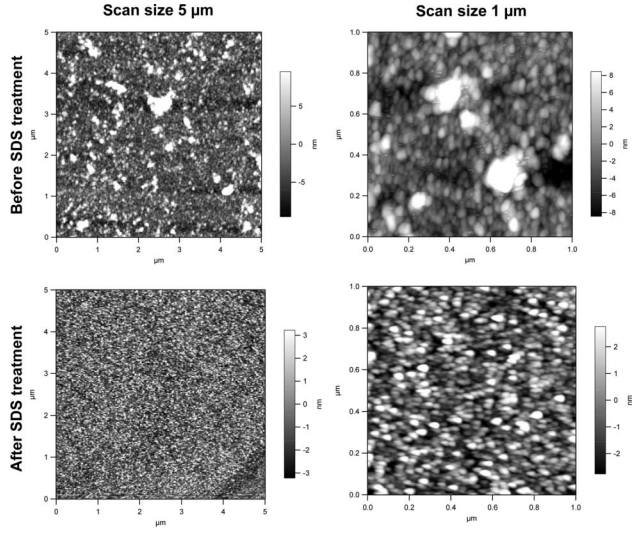

Figure 5. Representative AFM images (height plots) of glass surfaces coated with H*Protein A at 80°C for 16 h. The protein coating is shown before and after hot SDS treatment with scan sizes of 5 and 1 μm.

Impact of hydrophobin-modified surfaces on mature biofilms

To determine the impact of hydrophobin modifications on mature biofilms mixed bacterial populations were analysed. Effluent wastewater of a municipal sewage treatment plant with a known high bacterial density and diversity was used as inoculum. The biofilms were grown on bare glass surfaces and glass surfaces modified with H*Protein A and H*Protein B (before and after SDS treatment) in a biofilm flow through reactor. Synthetic wastewater was used as nutrient solution to keep the growth conditions constant.

After incubation for 4 weeks the bacteria were stained directly on the surfaces with the DNA intercalating dye Syto9 and the spatial distribution of the biofilms was analysed by epifluorescence microscopy. No significant differences in biofilm structure or spatial distributions were observed on the bare glass surfaces, H*Protein A-, and H*Protein B-modified glass surfaces (Figure 6). All surfaces were covered with multi-layered biofilms.

Besides the biofilm structure, the number of cultivable bacteria was determined. The colony-forming units were quantified by pour-plating on growth medium for heterotrophic bacteria (R2A medium). A number of 6×10^4 cultivable bacterial cells per cm^2 were present on unmodified glass surfaces (Table 2). The number of cultivable bacteria on hydrophobin-modified surfaces was in the same order of magnitude and ranged from 1×10^4 cells cm^{-2} on surfaces modified with H*Protein B before SDS treatment to 6×10^4 cells cm^{-2} on surfaces coated with H*Protein A before SDS treatment.

Microbial communities were analysed qualitatively *via* a molecular biology fingerprint method (Muyzer et al. 1993; Emtiazi et al. 2003). PCR-DGGE fingerprints were used to detect differences of the biofilm community compositions on bare and hydrophobin-coated glass surfaces. In addition, the bulk water was also analysed with regards to the planktonic communities. For the initial PCR reaction, the primer set 27F and 517R targeting the eubacterial 16S rDNA was applied. The 526 bp PCR products were separated by DGGE.

Firstly, the DGGE pattern of the bulk water was analysed (Figure 7). Thirty one DNA bands reflecting the number of different eubacterial species were counted and this confirmed the high bacterial diversity of the natural wastewater used. The different biofilm communities were not as diverse as the planktonic phase. Thirteen to 24 DNA bands were detected in the biofilm samples of the bare and hydrophobin-modified glass slides. Not all DNA bands present in the DGGE band pattern of the bulk water were found in the band pattern of the biofilm samples. The Dice coefficient (Cs) for the pair-wise comparison of microbial communities indicated the percentage of similarity (Table 3). The similarity of the bulk water community and the various biofilm samples was found to range between

Table 1. Surface coverage of bare and hydrophobin-coated glass surfaces with *E. coli* cells after incubation for 5, 17, and 26 h in a biofilm flow through reactor.

Incubation time (h)	Surface modification	Surface coverage (%)
17	Control	0.15 ± 0.22
	H*Protein B, before SDS treatment	0.39 ± 0.14
	H*Protein B, after SDS treatment	0.41 ± 0.32
26	Control	0.26 ± 0.18
	H*Protein B, before SDS treatment	0.33 ± 0.15
	H*Protein B, after SDS treatment	0.51 ± 0.26

Note: Surface coverage was averaged from four representative microscope images. ± = the SD.

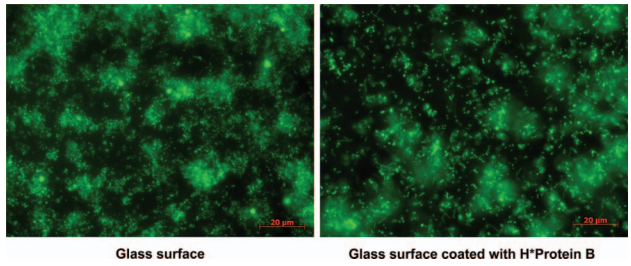

Figure 6. Representative fluorescence microscope images of mature biofilms formed on bare and H*Protein B (after SDS treatment)-modified glass surfaces. Natural wastewater was used for biofilm cultivation in a reactor fed with synthetic wastewater flow. The biofilms were analysed after 4 weeks and stained directly on the surfaces with the DNA intercalating dye Syto9.

Table 2. Number of cultivable bacteria per 1 cm^2 on bare and hydrophobin-modified glass surfaces after incubation for 4 week in a biofilm flow through reactor.

Surface modification	CFU cm^{-2}
Control	5.6×10^4
H*Protein A, before SDS treatment	6.4×10^4
H*Protein A, after SDS treatment	2.8×10^4
H*Protein B, before SDS treatment	1.3×10^4
H*Protein B, after SDS treatment	2.8×10^4

Note: The viable cell counts were determined in triplicate on growth medium for heterotrophic bacteria (R2A medium) after incubation for 7 days at 20°C. The mean SEs are in a range of 10–25%.

59% and 76% and confirmed a marginally selective colonisation. The percentage of similarity increased when comparing the biofilm populations from the bare and hydrophobin-coated glass surfaces. A similarity ranging between 75% and 85% was calculated, indicating no influence of the modified surface hydrophobicity on the microbial population composition.

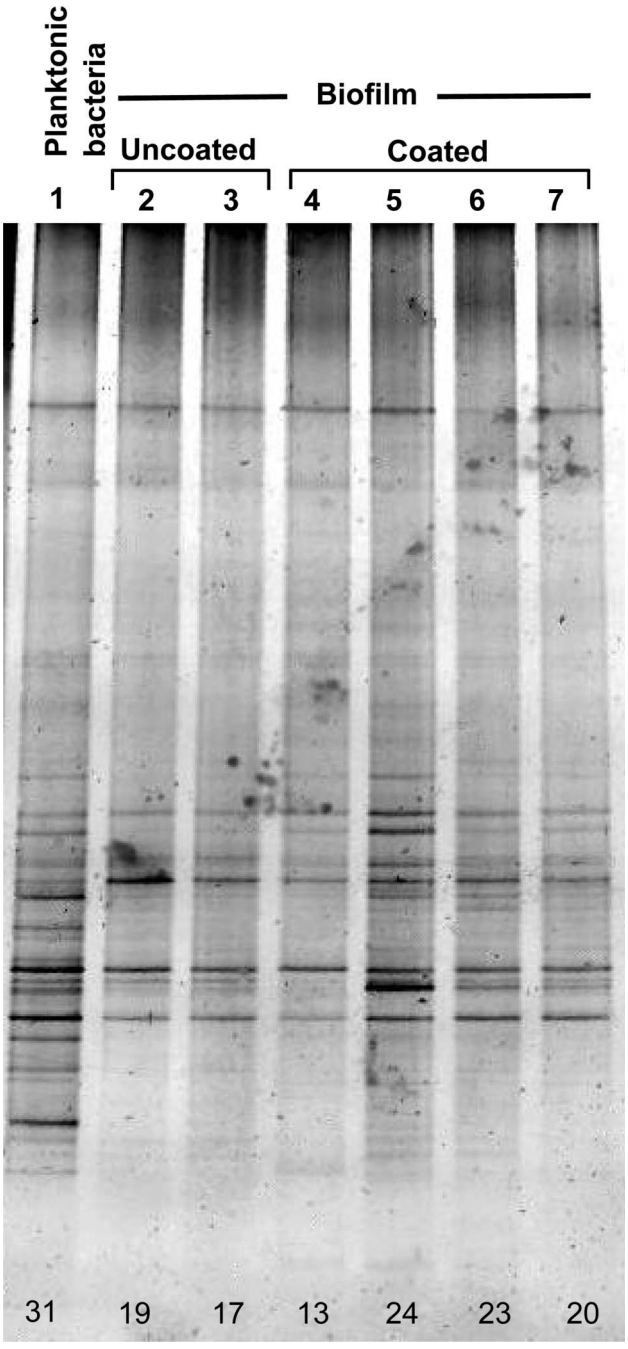

Figure 7. PCR-DGGE analyses of biofilm samples from different bare and hydrophobin-modified surfaces. Separated amplicons from planktonic bacteria from a bulk water sample are represented in lane 1. Lanes 2 and 3 = biofilms from bare glass surfaces; lanes 4 and 5 = biofilms from H*Protein A-modified surfaces before and after SDS treatment; lanes 6 and 7 = biofilms from H*Protein B-modified surfaces before and after SDS treatment. The numbers of DNA bands are given at the bottom of each lane.

Discussion

Hydrophobins are highly surface-active fungal proteins which are neither immunogenic nor toxic. They can be isolated from natural resources, eg *Schizophyllum commune* cultures in milligram amounts in a time-consuming process (Scholtmeijer et al. 2001). Recently, the BASF successfully established a recombinant production process for hydrophobins (Wohlleben et al. 2010). This process was based on the generation of two fusion-hydrophobins (H*Protein A and H*Protein B) consisting of the class I hydrophobin DewA of *Aspergillus nidulans*, the synthase yaaD of *Bacillus subtilis* in complete or truncated form, and a C-terminal His-tag. The fusion-partner yaaD was necessary for successful recombinant expression. Fermentation and downstream processing were up-scaled to pilot production on a kilogram scale. The structure formation and interface activity of the recombinantly produced hydrophobins H*Protein A and H*Protein B were not inhibited by the attached fusion-protein (Wohlleben et al. 2010). In the present study, the application of the recombinant fusion-hydrophobins for (large-scale) surface coating was tested with regard to changed surface properties and bacterial response (repulsion or attraction).

First, the qualitative and quantitative adsorption properties of the hydrophobins were determined using the QCM-D technique. The adsorption of H*Protein A and H*Protein B induced a change in dissipation of $\sim 1 \times 10^{-6}$ for a change in resonance frequency of 10–12 Hz. Usually, ΔD values for globular proteins are $\sim 1 \times 10^{-6}$ for a 20–40 Hz change in frequency when

Table 3. Comparison of PCR-DGGE fingerprints from different bacterial populations (biofilms and planktonic bacteria) by the Dice coefficient.

Sample	1	2	3	4	5	6	7
1	1.00						
2	0.76	1.00					
3	0.71	0.94	1.00				
4	0.59	0.75	0.80	1.00			
5	0.76	0.79	0.78	0.65	1.00		
6	0.74	0.81	0.85	0.67	0.89	1.00	
7	0.67	0.81	0.76	0.73	0.86	0.93	1.00

Note: 1 = planktonic bacteria; 2 and 3 = biofilms from glass surfaces; 4 = biofilms from H*Protein A-modified surfaces (before SDS treatment); 5 = biofilms from H*Protein A-modified surfaces (after SDS treatment); 6 = biofilms from H*Protein B-modified surfaces (before SDS treatment); 7 = biofilms from H*Protein B-modified surfaces (after SDS treatment).

they adsorb as a rigid layer (Hook et al. 1998a, 1998b, 1998c; Linder et al. 2002). The ratio of ΔD to Δf was higher for the recombinant fusion-hydrophobins and indicated that the proteins formed a soft layer on the hydrophilic SiO_2 surfaces. In contrast to this, natural hydrophobins like HFBI and HFBII from *Trichoderma reesei* adsorbed on SiO_2 surfaces in fairly rigid layers. They caused a change in frequency of up to 75 Hz, while the change in dissipation did not exceed 1.5×10^{-6} (Linder et al. 2002). Differences in the rigidity of the protein layers of the natural hydrophobins and the recombinant fusion-hydrophobins might be due to the yaaD fusion-protein exposed to the solution.

The QCM-D data demonstrated that the recombinant fusion-hydrophobins H*Protein A and H*Protein B adsorbed efficiently to the hydrophilic SiO_2 surfaces and formed 15–17 nm thick protein layers. These data were in agreement with AFM measurements of H*Protein B layers, according to which the average layer thickness was 12 nm at 70°C (Santhiya et al. 2010). Furthermore, 15–17 mg of the fusion-hydrophobins was calculated to coat 1 m^2. Of the smaller natural hydrophobins HFBI and HFBII (7.5 and 7.2 kDa, respectively), only 2.9–4.4 mg were needed to coat 1 m^2 in monolayer manner (Linder et al. 2002). This difference between recombinant and natural hydrophobins might be due to the large proportion of the fusion protein (20–50% of the total protein). Nevertheless, fusion-hydrophobins were highly suitable for large scale surface modification.

H*Protein A and H*Protein B self-assembled on hydrophilic glass surfaces and increased surface hydrophobicity in a temperature- and time-dependent manner. It is known that natural hydrophobins adhere to interfaces first in an intermediate α-helical conformation. This conformation and the film formed were not very stable and the hydrophobin could be dissociated from the surface with diluted detergents at room temperature (De Vocht et al. 2002). At elevated temperatures, by contrast, detergents induced the hydrophobin to proceed to a stable β-sheet end form remaining adsorbed to the solid surface. The detergent and the increased temperature might give the protein some flexibility and/or mobility permitting the transition to the β-sheet state (De Vocht et al. 2002). Hydrophobins adsorbed in the β-sheet end form resisted treatment with diluted detergents at room temperature, showing that this conformation interacted more strongly with the surface than the intermediate α-helical form (Janssen et al. 2004). The β-sheet form is important from a technological perspective since for many applications of hydrophobins, strong interactions with the surfaces are required.

To induce the proposed stable β-sheet end conformation, the fusion-hydrophobin coatings on the glass surfaces were treated with 2% SDS at 80°C. Due to this treatment, parts of the hydrophobin layer and the corresponding surface hydrophobicity were lost. Partial loss of the protein layer due to hot SDS treatment was also observed for natural hydrophobins. Treatment of teflon sheets coated with the *Schizophyllum commune* hydrophobins SC3 and SC4 resulted in 21% and 28% loss, respectively, at 20°C. For coatings at 80°C, only 15% and 7%, respectively, were lost (Janssen et al. 2004). This was also confirmed for the *A. nidulans* fusion-hydrophobins used in this study. In detail, the coatings which were performed at 80°C for 16 h were more stable and still homogenous after SDS treatment.

An important property of hydrophobins is their ability to change surface hydrophobicity. However, other proteins like bovine serum albumin (BSA) also adhere to various surfaces, and change the surface wettability and the surface tension. After adsorption of BSA and other proteins to hydrophobic FEP-Teflon the surface tension and the surface hydrophobicity were slightly decreased. Whereas, after adsorption of the *S. commune* hydrophobin SC3 a larger decrease in surface tension and an increase in contact angle was observed (Van der Vegt et al. 1996). On glass slides, which were coated with BSA according to the protocol described for 1, 6, and 16 h at 20°C, contact angles of 40–42° were measured (data not shown). In contrast to the surface modifications with recombinant fusion-hydrophobins, the surface hydrophobicity of BSA-coated surfaces was completely lost after 2% SDS treatment at 80°C. Surface coatings with other proteins like fibrinogen and γ-globulin also were washed off completely by the hot SDS treatment (Wösten et al. 1994). An outstanding characteristic of hydrophobin assemblages is their high stability. The results indicate that the assembly of the fusion-hydrophobins on the glass surfaces was caused by the specific binding of the hydrophobin DewA rather than the N-terminally attached fusion-protein, which would result in a complete loss of the protein layer as already described above.

The recombinant fusion-hydrophobins H*Protein A and H*Protein B self-assembled on hydrophilic glass surfaces and changed the contact angles to 73° and 71° before treatment and 71° and 42° after SDS treatment. On hydrophilic surfaces coated with natural hydrophobins, much higher water contact angles of 113° to 120° were measured (Zampieri et al. 2010). Recently, the hydrophobin DewA was expressed in the fungus *Hypocrea jecorina* as a recombinant hydrophobin without an additional N-terminal fusion-protein. The bioactivity of this recombinantly expressed DewA (10 kDa) was tested on glass as a hydrophilic reference substratum. Due to the hydrophobin coating, the contact angles of the glass surface were increased

considerably to 73° before and 70° after SDS treatment (Schmoll et al. 2010), which is in agreement with the present results. Therefore, the range of alteration in hydrophobicity seems to result from species specific properties of the hydrophobins.

The analysis of coating homogeneity with immunofluorescence and AFM confirmed the temperature- and time-dependent adsorption of the recombinant hydrophobins H*Protein A and H*Protein B. For a homogeneous coating after SDS treatment (proposed β-sheet conformation), a long incubation at an elevated temperature was necessary. SEM pictures of Teflon surfaces coated with the natural hydrophobins SC3 and SC4 of *S. commune* also indicated the influence of coating temperature on coating homogeneity (Janssen et al. 2004). On surface coatings produced at 80°C, treatment with hot SDS did not influence the surface morphology, whereas on surfaces coated at 20°C small pores were formed.

It was demonstrated that recombinant fusion-hydrophobins were well suited for large-scale surface coatings. They formed homogenous and stable protein monolayers on glass substrata, which directly influenced the surface hydrophobicity. Hydrophobic interactions are considered to have an impact on initial bacterial adhesion as these forces can act either in a repulsive or attractive manner (MacEachran and O'Toole 2007). Up to now, no effects of hydrophobin modifications on microbial systems were known. Therefore, this study was focused on biofilm formation regarding retardation, repulsion, and attraction. The influence of hydrophobin coatings on initial bacterial adhesion as well as maturing biofilms of *E. coli* and natural wastewater populations were analyzed.

Initial bacterial adhesion and linking film formation is the most critical step in biofilm formation (Busscher and Van der Mei 1997). Most cells within a biofilm structure rely on this interaction between the surface and the bacterial cells for their survival (Palmer et al. 2007). Surface properties like hydrophobicity, roughness and a predisposition to protein adsorption are recognized as important for bacteria to colonize a surface (Donlan 2002; Palmer et al. 2007). So far, the dominating factor involved in the initial adhesion of bacteria is unknown, but it is suggested that hydrophobic interactions might be responsible for much of the adherence of cells to surfaces (Hood and Zottola 1995). In biological systems, hydrophobic interactions are usually considered to be the strongest of all long-range non-covalent interactions (Van Oss 1997). Many studies suggest a correlation between hydrophobicity and cell attachment to surfaces (Van Loosdrecht et al. 1987; Gilbert et al. 1991; Peng et al. 2001; Iwabuchi et al. 2003; Liu et al. 2004). However, the specific effect of surface hydrophobicity and other parameters varied under different experimental conditions (Tang et al. 2011) because external factors like shear forces, flow rates and the bacterial strains themselves might have an influence on the results. This study showed that the change of surface wettability slightly enhanced the initial adhesion of *E. coli* cells to hydrophobin-modified glass surfaces in comparison to bare surfaces. Accordingly, the increased surface hydrophobicity as well as the adsorption of organic molecules could act as attractive forces altering the rates of bacterial attachment (MacEachran and O'Toole 2007). It has also been suggested that the presence of proteins on a surface favours biofilm formation, as attached proteins could be a source of nutrients for bacteria (Jeong and Frank 1994).

In contrast, *in vivo* and *in vitro* studies of oral cavities demonstrated that more microorganisms are attracted by high surface free energy (hydrophilic) substrata (Van Pelt et al. 1985; Quirynen et al. 1989). Next to hydrophobic interactions, which have an impact on initial bacterial attachment, the binding forces of bacteria play a role in detachment and biofilm stability. Increased detachment of bacteria in the oral cavity might be due to lower binding forces between bacteria and hydrophobic surfaces. Also, on hydrophobin-modified glass surfaces increased detachment of bacteria may occur under dynamic shear conditions, and this should be further investigated.

For the analysis of the effect of the hydrophobin coated surfaces on mixed mature biofilms, natural wastewater populations were used. Here, the interactions of different bacteria are expected to exhibit synergistic interactions and therefore have an impact on biofilm formation (Moons et al. 2009). Some bacterial species exhibit cell surface properties such as fimbriae, flagella, and surface-associated polysaccharides or proteins, which may provide an advantage in colonising surfaces with specific characteristics (Donlan et al. 2002). These bacteria can support the attachment and growth of other bacterial species in the biofilm. In this study, fluorescence microscopy determined the same spatial distribution of the biofilm on the hydrophilic glass surfaces and the hydrophobic (modified) surfaces. Moreover, the same number of viable bacteria adhering to the different surfaces was determined using plate counts. In addition to the binding forces between bacteria and low/high surface free energy surfaces, the selectivity in bacterial adhesion to hydrophobic/hydrophilic surfaces plays a role in biofilm formation. Busscher et al. (1984) showed that bacteria with a low surface free energy adhered in the highest numbers to low-energy solids, whereas bacteria with a high surface free energy adhered better to high-energy solids. Moreover, Weerkamp et al. (1989) demonstrated a selection of more hydrophobic strains from the same species adhering to hydrophobic

vs hydrophilic surfaces. With respect to the present data, the community composition on bare and hydrophobin modified glass surfaces was determined with PCR-DGGE of ribosomal DNA. With this technique no selection of different subpopulations was identifiable. The same bacterial populations adhered to the different surfaces. In consequence, surface wettability did not have any impact on biofilm formation by individual bacterial species in the mixed wastewater community.

To conclude the microbiological part of this study, biofilms were grown on bare and hydrophobin-coated glass surfaces. Surface wettability slightly influenced initial bacterial adhesion in pure culture under laboratory conditions, but no effects on the community composition (PCR-DGGE), cell number (cultivation), or spatial distribution (fluorescence microscopy) of natural mixed biofilms were found.

In future, recombinant fusion-hydrophobins are highly suitable for subsequent functionalization, where antimicrobial agents such as antimicrobial peptides, biocides or specific enzymes on surfaces are immobilised with hydrophobin monolayers to avoid or retard microbial biofilm formation.

Acknowledgements

This work was funded by a grant from the German Federal Ministry of Education and Research to T.S. as part of the research program 'Geotechnologien' (03G0723). The authors thank the BASF-SE (C. Bollschweiler and M. Karos) for providing the recombinant fusion-hydrophobins H*Protein A and H*Protein B.

References

Boe-Hansen R, Martiny AC, Arvin E, Albrechtsen HJ. 2003. Monitoring biofilm formation and activity in drinking water distribution networks under oligotrophic conditions. Water Sci Technol 47:91–97.

Busscher HJ, Van der Mei HC. 1997. Physico-chemical interaction in initial microbial adhesion and relevance for biofilm formation. Adv Dent Res 11:24–32.

Busscher HJ, Weerkamp, AH, Van der Mei HC, Van Pelt AWJ, De Jong HP, Arends J. 1984. Measurements of the free energy of bacterial cell surfaces and its relevance for adhesion. Appl Env Microbiol 48:980–983.

Costerton JW. 2002. Anaerobic biofilm infections in cystic fibrosis. Mol Cell 4:699–700.

Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322.

De Vocht ML, Reviakine I, Wösten HAB, Brisson A, Wessels JGH, Robillard GTJ. 2000. Structural and functional role of the disulfide bridges in the hydrophobin SC3. Biol Chem 275:28428–28432.

De Vocht ML, Reviakine I, Ulrich WP, Bergsma-Schutter W. Wösten HAB, Vogel H, Brisson A, Wessels JGH, Robillard GT. 2002. Self-assembly of the hydrophobin SC3 proceeds via two structural intermediates. Protein Sci 11:1199–1205.

De Vries OMH, Fekkes MP, Wösten HAB, Wessels JGH. 1993. Insoluble hydrophobin complexes in the walls of *Schizophyllum commune* and other filamentous fungi. Arch Microbiol 159:330–335.

Dexter SC, Sullivan JD, William J, Watson SW. 1975. Influence of substrate wettability on the attachment of marine bacteria to various surfaces. Appl Microbiol 30:298–308.

Donlan RM. 2002. Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890.

Emtiazi F, Schwartz T, Marten SM, Krolla-Sidenstein P, Obst U. 2003. Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration. Water Res 38:1197–1206.

Gilbert P, Evans DJ, Evans E, Duguid IG, Brown MRW. 1991. Surface characteristics and adhesion of *E. coli* and *Staphylococcus epidermidis*. J Appl Bacteriol 71:72–77.

Halthur TJ, Arnebrant T, Macakova L, Feiler A. 2010. Sequential adsorption of bovine mucin and lactoperoxidase to various substrates studied with quartz crystal microbalance with dissipation. Langmuir 26:4901–4908.

Hektor HJ, Scholtmeijer K. 2005. Hydrophobins: proteins with potential. Curr Opin Biotechnol 16:434–439.

Hood SK, Zottola EA. 1995. Biofilms in food processing. Food Control 6:9–18.

Hook F, Rodahl M, Brzezinski P, Kasemo B. 1998a. Energy dissipation kinetics for protein and antibody–antigen adsorption under shear oscillation on a quartz crystal microbalance. Langmuir 14:729–734.

Hook F, Rodahl M, Brzezinski P, Kasemo B. 1998b. QCM-measurements of ferritin monolayers on methyl-thiolated gold: dependence of energy dissipation and saturation coverage on salt concentration. J Colloid Interface Sci 208:263–267.

Hook F, Rodahl M, Kasemo B, Brzezinski P. 1998c. Structural changes in hemoglobin during adsorption to solid surfaces: effects of pH, ionic strength, and ligand binding. P Natl Acad Sci USA 95:12271–12276.

Iwabuchi N, Sunairi M, Anzai H, Morisaki H, Nakajima M. 2003. Relationships among colony morphotypes, cell surface properties and bacterial adhesion to substrata in *Rodococcus*. Colloids Surf B 30:51–60.

Janssen MI, van Leeuwen MBM, van Kooten TG, De Vries J, Dijkhuizen L, Wösten HAB. 2004. Promotion of fibroblast activity by coating with hydrophobins in the beta-sheet end state. Biomaterials 25:2731–2739.

Jeong DK, Frank JF. 1994. Growth of *Listeria monocytogenes* at 10 degrees C in biofilms with microorganisms isolated from meat and dairy processing environments. J Food Protect 57:576–586.

Linder M, Szilvay GR, Nakari-Setälä T, Söderlund H, Penttilä M. 2002. Surface adhesion of fusion proteins containing the hydrophobins HFBI and HFBII from *Trichoderma reesei*. Protein Sci 11:2257–2266.

Liu Y, Yang S, Li Y, Xu H, Qin L, Tay J. 2004. The influence of cell and substratum surface hydrophobicities on microbial attachment. J Bacteriol 110:251–256.

MacEachran DP, O'Toole GA. 2007. Do not fear commitment: the initial transition to a surface lifestyle by pseudomonads. In: Kjelleberg S, Givskov M, editors. The biofilm mode of life – mechanisms and adaptations. Norfolk (UK): Horizon Biosciences. p. 23–35.

Moons P, Michiels CW, Aertsen A. 2009. Bacterial interactions in biofilms. Crit Rev Microbiol 35:157–168.

Murray AE, Hollibaugh JT, Orrego C. 1996. Phylogenetic composition of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl Environ Microbiol 62:2676–2680.

Muyzer G, Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700.

Palmer J, Flint S, Brooks J. 2007. Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol 34:577–588.

Peng JS, Tsai WC, Chou CC. 2001. Surface characteristics of *Bacillus cereus* and its adhesion to stainless steel. Int J Food Microbiol 65:105–111.

Pringle JH, Fletcher M. 1983. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. Appl Environ Microbiol 45:811–817.

Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Arends J, Darius PL, van Steenberghe D. 1989. The influence of surface free-energy on planimetric plaque growth in man. J Dent Res 68:796–799.

Reasoner DJ, Geldreich EE. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 1:1–7.

Rosenhahn A, Ederth T, Pettitt ME. 2008. Advanced nanostructures for the control of biofouling: the FP6 EU Integrated Project AMBIO. Biointerphases 3(1):IR1–5.

Santhiya D, Burghard Z, Greiner C, Jeurgens LP, Subkowski T, Bill J. 2010. Bioinspired deposition of TiO2 thin films induced by hydrophobins. Langmuir 26:6494–6502.

Schmoll M, Seibel C, Kotlowski C, Wöllert Genannt Vendt F, Liebmann B, Kubicek CP. 2010. Recombinant production of an *Aspergillus nidulans* class I hydrophobin (DewA) in *Hypocrea jecorina* (*Trichoderma reesei*) is promoter-dependent. Appl Microbiol Biotechnol 88:95–103.

Scholtmeijer K, Wessels JG, Wösten HA. 2001. Fungal hydrophobins in medical and technical applications. Appl Microbiol Biotechnol 56:1–8.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85.

Tang L, Pillai S, Revsbech NP, Schramm A, Bischoff C, Meyer RL. 2011. Biofilm retention on surfaces with variable roughness and hydrophobicity. Biofouling 27:111–121.

Van der Mei HC, Rosenberg M, Busscher HJ. 1991. Assessment of microbial cell surface hydrophobicity. In: Mozes N, Handley PS, Busscher HJ, Rouxhet PG, editors. Microbial cell surface analysis: structural and physicochemical methods. New York: VCH. p. 263–287.

Van der Vegt W, Van der Mei HC, Wösten HAB, Wessels JGH, Busscher HJ. 1996. A comparison of the surface activity of the fungal hydrophobin SC3p with those of other proteins. Biophys Chem 57:253–260.

Van Loosdrecht M, Lyklema J, Norde W, Schraa G, Zehnder AJ. 1987. The role of bacterial cell wall hydrophobicity in adhesion. Appl Environ Microbiol 53:1893–1897.

Van Oss CJ. 1997. Hydrophobicity and hydrophilicity of biosurfaces. Curr Opin Colloid In 2:503–512.

Van Pelt AWJ, Weerkamp AH, Uyen MHW, Busscher HJ, De Jong HP, Arends J. 1985. Adhesion of *Streptococcus sanguis* CH3 to polymers with different surface free energies. Appl Environ Microbiol 49:1270–1275.

Weerkamp AH, Quirynen M, Marechal M, Van der Mei HC, Van Steenberghe D, Busscher HJ. 1989. The role of surface free energy in the early in vivo formation of dental plaque on human enamel and polymeric substrata. Microb Ecol Health D 2:11–18.

Wegerer A, Sun T, Altenbuchner J. 2008. Optimization of an *E. coli* L-rhamnose-inducible expression vector: test of various genetic module combinations. BMC Biotechnol 8:1–12.

Wessels JGH. 1997. Hydrophobins, proteins that change the nature of the fungal surface. Adv Microbial Physiol 38:1–45.

Wiencek KM, Fletcher M. 1997. Effects of substratum wettability and molecular topography on initial adhesion of bacteria to chemically defined substrata. Biofouling 11:293–311.

Wilms B, Wiese A, Syldatk C, Mattes R, Altenbuchner J, Pietzsch M. 1999. Cloning, nucleotide sequence and expression of a new L-N-carbamoylase gene from *Arthrobacter aurescens* DSM 3747 in *E. coli*. J Biotechnol 68:101–113.

Wohlleben W, Subkowski T, Bollschweiler C, von Vacano B, Liu Y, Schrepp W, Baus U. 2010. Recombinantly produced hydrophobins from fungal analogues as highly surface-active performance proteins. Eur Biophys J 39:457–468.

Wösten HAB, De Vocht ML. 2000. Hydrophobins, the fungal coat unravelled. Biochim Biophys Acta 1469:79–86.

Wösten HAB, De Vries O, Wessels J. 1993. Interfacial self-assembly of a fungal hydrophobin into a hydrophobic rodlet layer. Plant Cell 5:1567–1574.

Wösten HAB, Schuren FH, Wessels JG. 1994. Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. EMBO J 13:5848–5854.

Wösten HAB, Ruardy TG, Van der Mei HC, Busscher HJ, Wessels JGH. 1995. Interfacial self-assembly of a *Schizophyllum commune* hydrophobin into an insoluble amphipathic protein membrane depends on surface hydrophobicity. Colloids Surf B 5:189–195.

Yu L, Zhan B, Szilvay GR, Sun R, Jänis J, Wang Z, Feng S, Xu H, Linder MB, Qiao M. 2008. Protein HGFI from the edible mushroom *Grifola frondosa* is a novel 8 kDa class I hydrophobin that forms rodlets in compressed monolayers. Microbiology 154:1677–1685.

Zampieri F, Wösten HAB, Scholtmeijer K. 2010. Creating surface properties using a palette of hydrophobins. Materials 3:4607–4625.