Interfacial Rheology of Bacterial Biofilms

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ABSTRACT

The transient build-up of air-water bacterial biofilms of *Escherichia coli*, *Pseudomonas fluorescens*, and *Bacillus subtilis* is measured by interfacial rheology. The bacterial species had unique viscoelastic growth profiles, which were observed during growth conditions and under changing subphase conditions. As a complementary method, it may help to better understand the complex lifecycle of biofilms.

INTRODUCTION

Multicellular aggregates consisting of bacteria and extracellular matrix are called biofilms[1]. Biofilm formation on solid surfaces present a major issue in food and medical industries[2,3]. On the other hand, biofilms at the water-air interface are not commonly studied although several of these biofilm forming bacteria are potentially pathogenic[4].

Several biofilm measuring techniques have decisive disadvantages as they either measure biofilm strength indirectly or the measurements are invasive. In this contribution we utilize interfacial rheology to non-invasively study bacterial biofilms formed at the air-liquid surface under steady conditions. Additionally, with our modified setup[5], we are able to change the subphase conditions during measurements.

MATERIALS & METHODS

Materials

Three biofilm forming bacteria *Escherichia coli*, *Pseudomonas fluorescens*, and *Bacillus subtilis* were chosen and grown in LB (Lysogeny Broth). Stock cultures were frozen at – 70°C in glycerol 30 %(v/v) and obtained from various sources. The *E. coli* strain K12 csr (carbon storage regulator gene knock-out) and *P. fluorescens* SBW25 strain was obtained from the Institute of Biogeochemistry and Pollutant Dynamics (ETH Zürich, Switzerland). The *Bacillus subtilis* strain PY79 was obtained from the Institute of Integrative Biology (ETH Zürich, Switzerland). The *Bacillus subtilis* surfactin knockout NCIB 3610 was obtained from Kolter Laboratory (Harvard, USA). Working cultures were grown from the stock cultures by inoculating Mc-Cartney bottles at 1% (v/v) containing LB broth and incubated at 37°C for 24 h shaking at 160 rpm. Fresh medium was inoculated with 1 %(v/v) with this subculture and immediately used for subsequent measurements. All media were prepared with deionized water and sterilized by autoclaving at 120°C for 15 minutes.

Interfacial rheology

To test the transient build up of the biofilm formation at the water-air interface,

a shear rheometer (Phyisca MCR501, Anton Paar) with a biconical disk geometry was used (see Figure 1A). During interfacial rheological measurements, the interfacial flow is assumed to be decoupled from the bulk phase flow when the Boussinesq number (Bo) is >>1. Through sinusoidal oscillations with a defined deformation, a stress response can be measured. The resulting interfacial storage (G') and interfacial loss (G'') moduli were used to characterize the rheological behavior at the interface.

The bacteria were incubated inside the modified measuring cell. During the biofilm build up (t < 70 h), time sweeps were performed at a constant frequency and deformation (γ_0 = 0.1 % and ω = 1 s^{-1}) to observe structural changes. Unless stated otherwise, measurements were performed at 25°C. In a next step, the subphase was modified through a previously constructed device, which allowed simultaneous pH control during interfacial rheological measurements[5].

Tensiometry

To measure the surface tension over time of the biofilm formation, a pendant drop tensiometer (PAT-1, Sinterface technologies, Germany) was used (see Figure 1B). A detailed description of the measuring principles is given elsewhere[6]. In short, a drop is formed at the end of a capillary and monitored with a video camera. The Young-Laplace equation is used to fit the resulting drop contour. At a constant drop size the transient surface tension $\sigma(t)$ is measured. Measurements were performed at 20°C.

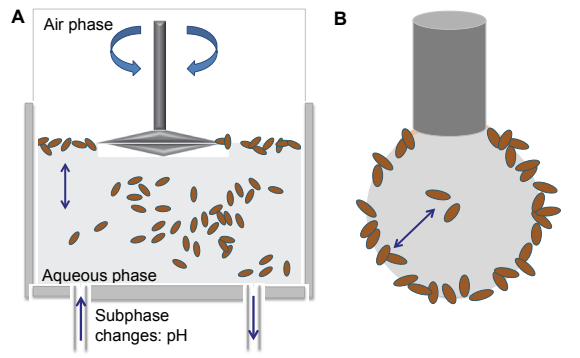

Figure 1. A: Schematic overview of subphase controlled interfacial rheological setup used for the bacterial biofilm elasticity measurements. B: Schematic of pendant drop tensiometer with a biofilm

RESULTS

Constant subphase conditions

The bacteria, *P. fluorescens* and *E. coli* were grown at 25°C in the interfacial rheological setup. Both bacteria exhibited biofilm formation at the interface as the elasticity increased over time. The first elasticity plateau observable in the elastic growth curves in Figure 2 represent the typical protein adsorption curve as a full media was used for bacterial growth (zone A in Figure 2).

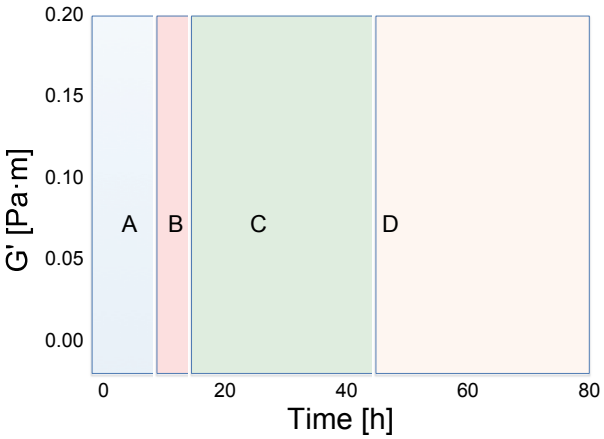

Figure 2. Elastic modulus growth profile of *E. coli* and *P. fluorescens* in LB media at 25°C.

The following decrease of elasticity is caused by the metabolism of the bacterium.

Through this metabolism, glucose present in the media is used up and the resulting acidification causes a decrease of elasticity (zone B). This was proven by pH measurements (shown later). After 15 - 20h, the biofilm elasticity increased in strength due to proteolytic activity releasing ammonia into the media and thus causing an increase of pH (zone C).

In Figure 2 the elasticity decreases after reaching an elasticity plateau (zone D). This mechanism can be explained by the biofilm lifecycle[1]. Through starvation, as no new media is introduced, the bacteria start to degrade their own biofilm. The elasticity however will never decrease to zero, as proteins are mostly irreversibly adsorbed at the interface. In the case of *E. coli* biofilms, a similar dynamic elastic behavior can be observed. Here a twofold rise in elasticity (42 and 70 h) is observed over time. This behavior shows how dynamic biofilm behavior differs greatly from constant elasticity values of proteins at interfaces.

In Figure 3, the time sweep of *B. subtilis* and a surfactin knockout mutant *B. subtilis* is shown. In the first 15 hours protein adsorption can be observed for both measurements.

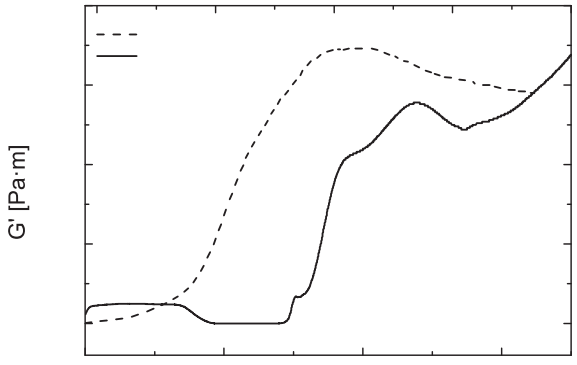

Figure 3. Elastic modulus growth profile of *B. subtilis* and *B. subtilis surfactin mutant* in LB media at 25°C.

After t > 15 h a sharp decrease of elasticity was observed for the non-mutant strain. The mutant strain showed an increase in elasticity in the same observed time frame. This sharp decrease of elasticity is caused by the production of surfactin by *B. subtilis*. Surfactin is a well known biosurfactant which can have antibacterial effects. The production of surfactin allows *B. subtilis* to spread on the water-air interface by lowering the surface tension[7]. At t = 15 hours, the surfactants displace the proteins as they lower the surface tension more efficiently[8,9]. The replacement causes a decrease of surface tension. After t > 30 h, the biofilm elasticity increases with time and an elastic biofilm is formed. With the help of surfactin, *B. subtilis* formed a stronger and thicker biofilm than *P. fluorescens* and *E. coli*.

The biofilm formation by *E. coli*, *P. fluorescens*, and *B. subtilis* were observed macroscopically and microscopically. All three bacteria formed biofilms with different morphologies and structures. Slimy textures (*P. fluorescens*), crumbly biofilms (*E. coli*) and thick layer biofilms (*B. subtilis*) were detected.

The surface tension was measured for *B. subtilis* and mutant *B. subtilis* strain as a function of time (see Figure 4).

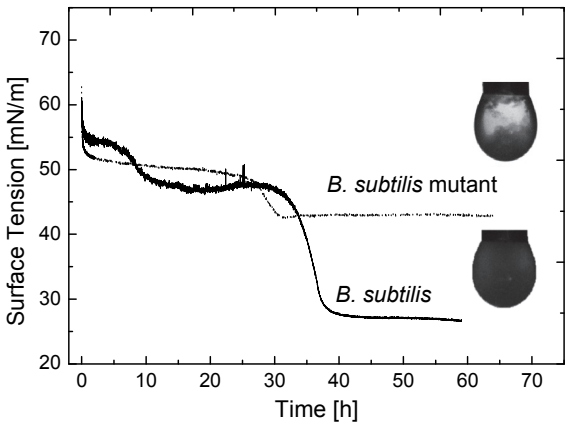

Figure 4. Surface tension measurement over time of *B. subtilis* and *B. subtilis* mutant in LB media.

The first plateau of surface tension is reached after 20 h and this value corresponds to the typical surface tension

values of adsorbed proteins. After t > 20 h the surface tension is lowered by surfactin. The strong decrease was not observed for the non-surfactin producing strain. The slight decrease, which can be observed can be caused by a small amount of produced molecules which can have an amphiphilic character. The lack of surfactin production could be observed visually as this mutant was unable to form a uniform biofilm over the whole surface area.

Changing subphase conditions

Biofilms are removed in industries through surfactants, disinfectants, and acid/base combinations[11]. The pH is important for bacterial growth and metabolism and is decisive tuning factor for the physical-chemical properties of proteins. To investigate the influence of pH on the pellicle, hydrochloric acid (HCl) at a concentration of 0.25 M was injected into the subphase. The pH change simulates a changing environmental condition after bacterial biofilm formation (see Figure 5).

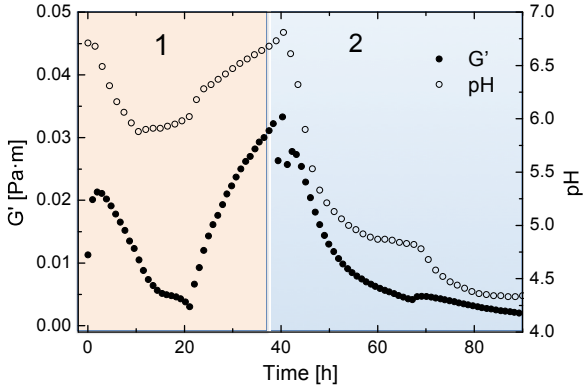

Figure 5. Elasticity growth profile and pH of *E. coli* against time in LB media at 25°C.

In a first step, the biofilm was allowed to grow under constant subphase conditions (t < 40 h, zone I). For all bacteria a strong dependency between pH and elasticity was observed. The first few hours (10-15 hours) protein and bacterial adsorption occurs. The decrease of elasticity is caused by the natural acidification of the media through the bacterial metabolism. The following rise of elasticity is caused by the proteolysis of LB. The increasing pH leads to an increase of elasticity through an increase of attractive forces between protein molecules.

After biofilm formation, the pH was lowered to a pH 4 – 5 and this lead to a decrease of elasticity of the biofilm (t > 40h, zone II). Thus, the network elasticity is strongly dependent on pH. A pH decrease leads to a decrease of attractive forces and an increase of repulsive forces. This strong dependency of elasticity and pH was observed in a recent study about network elasticity of amyloid fibrils at water-oil interfaces[5]. Since the investigated biofilms contain amyloid fibrils as structural elements, the influence of pH on the fibers can not be neglected [4,12,13]. The pH also has a strong influence on the bacterial metabolism and attachment.

Besides mechanical treatments and the use of acid chemicals, surfactants are used to remove biofilms[14]. To test the effect of surfactants, non-ionic Tween 20 was introduced into the subphase. Tween 20 molecules are small and adsorb quickly to the interface and intercalate in the existing protein network. On the one hand, they can solubilize protein into the subphase, on the other hand they can displace protein from the interface and lower the surface tension more efficiently[15,16,17].

Adding Tween 20 several times to the existing biofilm of *P. fluorescens* and *B. subtilis* is depicted in Figure 6. At first, a sharp decrease of elasticity can be observed for *P. fluorescens* biofilms. However, the biofilm elasticity seems to recover after some time and does not seem to be strongly affected by further additions.

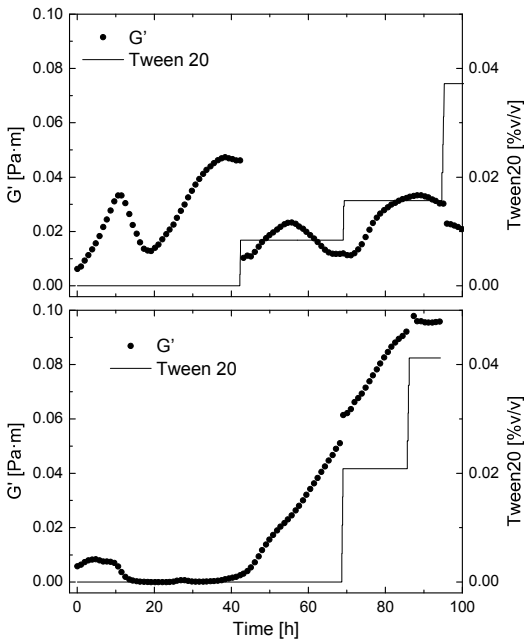

Figure 6. Interfacial elastic modulus of *P. fluorescens* (top) and *B. subtilis* (bottom) and against time before and after the addition of Tween20.

The elasticity however never really decreased completely. The network, containing other entangled macromolecules, prevents it from being removed from the interface. In comparison, *B. subtilis* biofilms were not affected after injection of Tween 20 as depicted in Figure 6. The biofilms by *B. subtilis* form very ordered structures and thus no free interface is available for the intercalation of Tween 20 as a nucleation point in the adsorption layer for more surfactants.

CONCLUSION

The suitability of interfacial rheology was tested to monitor pellicle formation at the air-liquid interface. Three biofilm forming bacteria, *E.coli*, *P. fluorescens*, and *B. subtilis*, were chosen and grown in the measuring devices at 25°C in Lysogeny Broth (LB) media. With interfacial rheology, the elasticity, viscosity and surface tension was measured over time. Through our elaborate setup, the measurements could be performed while simultaneously controlling the subphase conditions.

Characteristic elastic growth profiles due to the bacterial biofilm lifecycle could be observed for all bacteria. The strongest biofilm former was *B. subtilis* as very thick pellicles were formed by this bacterium. The elastic growth profile of *B. subtilis* also showed an interesting behavior as non-measurable values were recorded during growth caused by surfactin. This was proven by measuring a *B. subtilis* strain without the capability to produce surfactin.

By lowering the pH, the biofilms' elasticity can become not measurable as the dependency between pH and network elasticity was found to be strong. At pH values around 4 the biofilm network was completely disintegrated. The injection of Tween 20 to the subphase after biofilm formation hardly affected *B. subtilis* biofilms. The elasticity of *P. fluorescens* biofilm weakened initially but then showed a recovery effect of elasticity.

Interfacial rheology proved to be an effective method to measure biofilm formation online at the air-liquid interface. Through the observation of characteristic dynamic elasticity profiles of each bacterium and in combination with pendant drop tensiometry we were able to gain further insights into the still poorly understood mechanical properties of biofilms.

In comparison to bioassays, this method allows a quantitative observation over time without biofilm destruction. These measurements show that the transient elasticity behavior is highly dependent on bacteria type and media.

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