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Studying bacterial hydrophobicity and biofilm formation at liquid–liquid interfaces through interfacial rheology and pendant drop tensiometry

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ABSTRACT

Bacterial adsorption to interfaces is a key factor in biofilm formation. One major limitation to understanding biofilm formation and development is the accurate measurement of bacterial cell adhesion to hydrophobic interfaces. With this study, bacterial attachment and biofilm growth over time at water–oil interface was monitored through interfacial rheology and tensiometry. Five model bacteria (*Pseudomonas putida* KT2442, *Pseudomonas putida* W2, *Salmonella typhimurium*, *Escherichia coli*, and *Bacillus subtilis*) were allowed to adsorb at the water–oil interface either in their non-growing or growing state. We found that we were able to observe the initial kinetics of bacterial attachment and the transient biofilm formation at the water–oil interface through interfacial rheology and tensiometry. Electrophoretic mobility measurements and bacterial adhesion to hydrocarbons (BATH) tests were performed to characterize the selected bacteria. To validate interfacial rheology and tensiometry measurements, we monitored biofilm formation utilizing both confocal laser scanning microscopy and light microscopy. Using this combination of techniques, we were able to observe the elasticity and tension development over time, from the first bacterial attachment up to biofilm formation.

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1. Introduction

Complex multicellular assemblies such as bacterial biofilms are ubiquitous throughout nature and occur between a range of different interfaces (e.g. liquid–solid, liquid–air and liquid–liquid). Bacteria live in biofilms, their predominant life form, to multiply and protect themselves from environmental stresses such as UV radiation and osmotic shock [1–4]. Bacterial biofilms undergo several stages of development, from the initial attachment of bacteria to detachment and colonization of new interfaces [1,5,6]. The viscoelastic matrix can be composed of extracellular DNA, polysaccharides, and proteins [7,8]. Most biofilms of interest are undesirable as they form in drinking water pipelines, hulls of ships, and industrial surfaces causing economical drawbacks [3,9,10]. They are also known to cause food poisoning outbreaks and are common in clinical infections with severe consequences [11–13]. Improperly cleaned surfaces in the food industry with oil and protein residues are potential biofilm formation locations [14]. Additionally, bacteria are able to form bacterial adsorption layers or biofilms inside emulsion systems [15,16].

However, biofilms can have positive effects in waste water treatments and are key players in global ecology [17,1]. In bioremediation, bacteria are used to degrade alkanes and polycyclic aromatic compounds into harmless by-products [18–20]. Bacteria, forming water–oil biofilms, have a very diverse metabolism which allows them to use crude oil as a carbon source and form a biofilm around the oil droplets to enhance carbon uptake [6,21]. Furthermore, biofilms of probiotic bacteria in the gastrointestinal tract form a protective layer against invasive pathogenic bacteria [22,23]. Probiotic bacteria, for example, are not only screened for their health benefits but also for their biofilm forming abilities as this is involved in adhesion in the host [22–25]. The effectiveness of biofilm formation is dependent on the cell surface hydrophobicity, as the hydrophobicity is important in the initial attachment process of bacteria to hydrophobic surfaces [23,11].

Adhesion of bacteria to surfaces can occur through specific or unspecific binding. In unspecific binding, the initial step of bacterial adhesion can be described by the modified XDLVO (Derjagiun, Landau, Verwey, Overbeek) theory, which includes electrostatic, short range Lewis acid–base, and hydration interactions [26]. Under

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most physiological conditions the electrostatic interactions are repulsive, as both the bacteria and most surfaces are net negatively charged. However, due to the hydrophobicity of the complex and heterogeneous cell surface containing flagella, fimbriae, pilli, proteins, and surface polysaccharides, this repulsion is overcome. Numerous factors influence bacterial adhesion including pH, ionic strength, bacterial mobility, state of growth, and growth factors in the medium [12]. To study the adhesion of bacteria the classical approaches are: electrophoretic mobility to measure the electrostatic interactions, contact angle measurements, and the bacterial adhesion to hydrocarbons (BATH) test [27–29]. BATH tests, performed for the last 20 years, consists of a simple laboratory test where the adsorption of bacteria is measured before and after mixing with a hydrophobic phase [27,30]. The results from the literature can hardly be compared, as different hydrophobic phases, deposition times, pH's, and ionic strength can all have an influence on the hydrophobicity [31,32].

More sophisticated methods use biological adhesion assays and biofilm formation to hydrophobic surfaces such as polystyrene or to human intestine cells (Caco-2 cells) [33,23]. These tests only provide, as the BATH test, a relative percentage of hydrophobicity and biofilm formation. During biofilm formation, the different stages of biofilm development are hard to measure and are often missed. Confocal microscopy is able to reveal structural changes, however fails to give a quantitative value about cohesive strength. Other sophisticated methods, such as atomic force microscopy and microrheology, measure the cohesive strength of biofilms, however these are difficult to perform and are most probably not representative due to the heterogeneous nature of biofilms [34–36]. Real time changes in biofilm structure are therefore hardly captured with these methods, as they only describe certain development stages or measure biofilm strength indirectly.

To address these shortcomings, we used interfacial rheology and pendant drop tensiometry to register biofilm formation at the water–oil interface of biofilm forming bacteria, such as *Pseudomonas putida*. Additionally, relevant food industry bacteria (*Escherichia coli*, *Salmonella typhimurium*) and a model strain (*Bacillus subtilis*) were also measured. Interfacial rheology is conventially performed to characterize a large variety of soft matter material ranging from particles, proteins to surfactants [37–40] while biofilm structures at liquid–air interfaces have only been recently studied [41–43]. Pendant drop tensiometry was also used, as the interfacial tension decrease is a direct link to the hydrophobicity of the bacterial cells. We first characterized our bacteria with electrophoretic mobility measurements and light microscopy. The stationary (non-dividing) bacteria were observed at the water–oil interface through tensiometry and interfacial rheology. BATH tests were then performed to calculate the hydrophobicity values and correlate these to the decrease of interfacial tension. By performing strain sweeps, the network connectivity and resistance to strain was tested. To understand the consequence of the initial attachment to the oil interface, longterm observations were performed of non-stationary (dividing) bacteria with interfacial rheology and confocal laser scanning microscopy (CSLM). By combining a range of different approaches we were able to accurately characterize different biofilms, finding a large difference between species. By observing these differences, we were able to observe that interfacial rheology can be used to study bacterial adsorption and biofilms at liquid–liquid interfaces.

2. Materials and methods

2.1. Bacterial strains

Model bacteria such as (*Pseudomonas putida* KT2442, *Pseudomonas putida* W2, *Escherichia coli* K12, *Salmonella typhimurium*, and *Bacillus subtilis*) were chosen for this study and cultured in Lysogeny Broth (LB). Stock cultures frozen at −70 °C in glycerol 30% (v/v) were obtained by various sources. The *E. coli* K12 strain and *S. typhimurium* M556 strain were obtained from the Institute of Biogeochemistry and Pollutant Dynamics (ETH Zürich, Switzerland). The *P. putida strains* KT2442 and W2 were obtained from the Institute of Plant Biology (University of Zürich, Switzerland). Working cultures were grown from the stock cultures by inoculation 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. The medium were prepared with deionized water and sterilized by autoclaving at 120 °C for 15 min. For the bacterial cell suspensions, a phosphate buffer solution of pH 7 and a ionic strength of 100 mM was prepared (Sigma-Aldrich, Switzerland). As an oil phase, mineral oil (light, Sigma-Aldrich, Switzerland) and MCT (medium chain trigylcerides, Delios GmbH, Switzerland) oil were used. For comparative reasons n-hexadecane (Sigma-Aldrich, Switzerland) was used for BATH test.

2.2. Bacterial cell purification and characterization

To study the adsorption of bacterial cells at the water–oil interface, bacterial cells were isolated from the growth medium [44]. Fully grown cultures were taken (24 h at 30–37 °C) and centrifuged at 9500 × *g* for 2 min. The cells were resuspended in phosphate buffer (pH 7, I = 100 mM) after every centrifugation step. The obtained cell suspensions were measured with a photometer (BioPhotometer, Eppendorf, Germany) and the cell density adjusted to 0.6 at OD_{600} by diluting with the buffer solution.

To quantify the available surface charges on the bacterial surface, the electrophoretic mobility of the bacteria suspensions were measured. The electrophoretic mobility was measured using a Zetasizer (Nano series, Malvern Instruments) through laser Doppler velocimetry. The zeta potential ζ can be calculated in aqueous medium and moderate electrolyte concentration with the Smoluckowski equation:

$$\zeta = \frac{\eta\mu}{\varepsilon_0 \varepsilon} \tag{1}$$

where μ is the electrophoretic mobility, ε_0 the permitivity of vacuum, ε is the dielectric constant of the medium, and η the viscosity of the medium.

The bacterial adhesion to hydrocarbons (BATH) test was performed with a modified protocol of those described by Rosenberg et al. [27]. The cell suspension was mixed with the hydrophobic phase with a ratio of 1–1.2 and vortexed for 2 min. The resulting emulsion was left to separate for 15 min. The light absorbance of the aqueous phase was determined at 600 nm. The difference before $OD_{600,0}$ and after mixing $OD_{600,t}$ was used to calculate the bacterial adhesion as a percentage:

$$\mathrm{BATH}\,(\%) = 100 \times \left(1 - \frac{OD_{600,t}}{OD_{600,0}}\right) \tag{2}$$

In our case, we varied the hydrophobic phase by using n-hexadecane, mineral oil, and MCT oil.

2.2.1. Interfacial rheology

To investigate the transient build up of the bacterial adsorption layers and biofilm formation at the water–oil interface, a shear rheometer (Physica MCR300 and MCR501, Anton Paar) with a biconical disk geometry was used (see Fig. 1A). A more detailed methodology on interfacial rheology using the biconical disk geometry is presented in the literature [45].

In brief, for interfacial rheology the disk rheometer can be treated as a 2D Couette device when the interfacial flow is

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