Retention of bacteria on a substratum surface with micro-patterned hydrophobicity

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Abstract

Bacteria adhere to almost any surface, despite continuing arguments about the importance of physico-chemical properties of substratum surfaces, such as hydrophobicity and charge in biofilm formation. Nevertheless, in vivo biofilm formation on teeth and also on voice prostheses in laryngectomized patients is less on hydrophobic than on hydrophilic surfaces. With the aid of micro-patterned surfaces consisting of 10-μm wide hydrophobic lines separated by 20-μm wide hydrophilic spacings, we demonstrate here, for the first time in one and the same experiment, that bacteria do not have a strong preference for adhesion to hydrophobic or hydrophilic surfaces. Upon challenging the adhering bacteria, after deposition in a parallel plate flow chamber, with a high detachment force, however, bacteria were easily wiped-off hydrophobic lines, most notably when these lines were oriented parallel to the direction of flow. Adhering bacteria detached slightly less from the hydrophilic spacings in between, but preferentially accumulated adhering on the hydrophilic regions close to the interface between the hydrophilic spacings and hydrophobic lines. It is concluded that substratum hydrophobicity is a major determinant of bacterial retention while it hardly influences bacterial adhesion. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Over the past two decades, bacterial adhesion to substratum surfaces with different hydrophobicities has been studied by an enormous number of research groups [1–4]. Whereas it is sometimes concluded that bacterial adhesion is less to hydrophobic substrata [5], opposite conclusions are also drawn [6]. The diversity in the bacterial world is a factor contributing to this confusion, particularly since bacterial cell surfaces can be as hydrophilic as glass while other strains can have cell surfaces as hydrophobic as wax [7]. Recently, however, we have become convinced that this confusion is largely due to inadequate experimental systems to study bacterial adhesion [8]. In a typical ‘Materials and methods’ section of a paper on bacterial adhesion, it can be read that ‘substrata with adhering bacteria were slightly rinsed under running tap water’ or ‘dipped to remove loosely adhering bacteria prior to enumeration’.

Such methodology has been (and still is in many research groups) general practice for many years, but nevertheless is inadequate as it raises a number of obvious questions: (a) what is the magnitude of the rinsing forces applied, (b) what is the definition of ‘loosely adhering’ bacteria, (c) what percentage of the total adhering population is removed by rinsing?

To avoid these questions, we have started doing bacterial adhesion experiments in a parallel plate flow chamber under controlled hydrodynamic conditions [8]. With the aid of phase-contrast microscopy, ultra-long working distance objectives and image analysis, bacterial adhesion could be observed ‘live’, as it occurred and enumeration was instantaneous under the shear conditions of the actual experiment. Based on flow chamber studies involving several bacterial strains and substratum materials, it became increasingly difficult to conclude whether bacterial adhesion was more or less extensive on hydrophobic or hydrophilic substrata and it was often impossible to assign any significance to trends observed.

As always, ‘the proof of the pudding is in the eating’ and for us the pudding was the human oral cavity and the
2.1. Bacterial growth conditions

Streptococcus sobrinus HG1025 was cultured in Todd Hewitt broth at 37°C in ambient air. For each experiment, strains were inoculated from blood agar in a batch culture. This culture was used to inoculate a second culture which was grown for 16 h. Bacteria were harvested by centrifugation (5 min at 10,000×g), washed twice with demineralized water and finally suspended in a 10-mM potassium phosphate solution (pH 6.8) to a concentration of $3 \times 10^8$ ml$^{-1}$ for use in the parallel plate flow chamber.

2.2. Micro-patterning

Micro-patterned surfaces on glass were prepared by a lithographic technique previously described by Healy et al. [13]. Briefly, cleaned glass plates were spin-coated with a layer of photoresist (S-1813, Shipley, Marlborough, MA, USA) and subsequently exposed to light through a photomask. Next, the exposed resist was dissolved away in an alkaline solution and rinsed with water, baked at 100°C and stored. Finally, the plates were coated with dimethyl dichlorosilane (DDS) after which the undeveloped photoresist was removed by sonication in acetone. The result was a chemical pattern consisting of 10-μm wide, hydrophobic lines of DDS with 20-μm hydrophilic spacings in between. Water contact angles on similarly prepared DDS coatings on glass were about 90°, while glass had a water contact angle of around 20°. In addition, ellipsometry and X-ray photoelectron spectroscopy indicated that the thickness of such a DDS coating was 1–4 nm [13]. The micro-patterned surfaces could be inserted into the parallel plate flow chamber with the pattern either parallel or transverse to a flowing bacterial suspension [10]. The position of the hydrophilic lines was identified microscopically during the actual adhesion experiment as described below.

2.3. Parallel plate flow chamber

Deposition was observed on the bottom plate of the parallel plate flow chamber with a CCD-MXR camera (High Technology, Eindhoven, The Netherlands) mounted on a phase-contrast microscope (Olympus BH-2) equipped with a 40× ultra-long working distance objective (Olympus ULWD-CD Plan 40 PL). The camera was coupled to an image analyzer (TEA, Difa, Breda, The Netherlands). Live images were Laplace-filtered after subtraction of an out of focus image. Thereafter, adhering bacteria were discriminated from the background by single gray value thresholding. In this set up, one image covers a surface area of 0.016 mm$^2$. The internal dimensions of the parallel plate chamber were $3.8 \times 5.6 \times 0.06$ cm (width × length × height) and fluid flow was 1.5 ml min$^{-1}$.

All experiments were carried out in duplicate with separately cultured bacteria and newly prepared patterned surfaces. In each experiment, images were taken of three different locations on the patterned surface prior to challenging the adhering bacteria with the high detachment force exerted by a passing liquid–air interface, and of three
different locations after the exposure of the adhering bacteria to a passing liquid–air interface. The liquid–air interface was created by introducing an air-bubble in the flow chamber, that completely spanned the width of the chamber.

The position of the hydrophobic lines could be discerned from the de-wetting pattern after the passage of the liquid–air interface.

3. Results and discussion

Fig. 1 presents images of *S. sobrinus* HG1025 adhering from a flowing suspension to patterned surfaces. In the images taken prior to the passage of an air-bubble through the flow chamber, the micro-pattern can hardly be discerned, demonstrating that these streptococci do not have a strong preference for adhesion to either hydrophobic or hydrophilic substrata. Retention of the adhering streptococci, as observed in the images after their exposure to a liquid–air interface, clearly reveals the micro-pattern reflected in the density of adhering bacteria able to withstand this detachment force with slightly fewer bacteria adhering to the hydrophobic lines than to the hydrophilic spacing and with a preferential retention of bacteria on the hydrophilic spacings close to the interface between the hydrophilic and hydrophobic regions. Interface regions parallel to the flow more distinctly reveal the micro-pattern from the density of adhering bacteria than lines transverse to the flow.

Quantitative enumeration of the number of adhering streptococci prior to and after exposure high shear-off forces in Fig. 2 confirms the conclusion drawn from the images presented in Fig. 1, and prior to the passage of a liquid–air interface similar numbers of bacteria are found adhering over the different substratum regions (on average $1.8 \times 10^6$ cm$^{-2}$). After the passage of a liquid–air interface in a direction parallel to the patterning, up to $7 \times 10^6$ cm$^{-2}$ bacteria remain adhering on the hydrophilic spacings, close to the interface region, while less than $0.5 \times 10^6$ cm$^{-2}$ are found on the hydrophobic lines. When the hydrophobic lines are transverse to the flow and the direction of liquid–air interface passage, the effects of the micro-patterned hydrophobicity on bacterial retention are less pronounced.

The use of micro-patterned surfaces here for the first time in one and the same experiment demonstrates that bacterial adhesion is hardly influenced by substratum hydrophobicity, while substratum hydrophobicity is a major determinant of bacterial retention. The observation that a

![Fig. 1. Distribution of streptococci adhering on micro-patterned surfaces with hydrophobic lines on hydrophilic glass from a flowing suspension. Images were taken after 4-h exposure of the surfaces to flow; for the top panel (a and b), the hydrophobic lines were oriented parallel to the flow, while for the bottom panel (c and d), the lines were transverse to the flow; the distribution shown in the left-hand panel was taken prior to exposure of the adhering bacteria to a high shear-off force and the right-hand panel shows those bacteria that remained adhering after challenge by a liquid–air interface. Arrows indicate 10-µm wide hydrophobic lines.](https://academic.oup.com/femsle/article-abstract/189/2/311/525637)
relatively large number of bacteria remain adhering close to the interface between hydrophilic and hydrophobic regions after exposure to high shear forces is most interesting. This can possibly be explained by the detachment forces arising from the passing liquid–air interface, which more readily cause detachment from hydrophobic than from hydrophilic regions due to different adhesive forces and spontaneous de-wetting of the hydrophobic surface [14]. Consequently, when the pattern is parallel to the moving liquid–air interface, uninterrupted de-wetting occurs whereas in the alternative orientation de-wetting is repeatedly interrupted resulting in two distinct bacterial retention patterns. In addition, since bacteria are equipped with a variety of different binding structures that can either be hydrophobic or hydrophilic, accumulation of bacteria near the interface between hydrophilic and hydrophobic regions might be a result of the most optimal interaction between the heterogeneous bacterial cell and the substratum surface. This speculation is supported by observations that mammalian cells and also proteins preferentially accumulate on the most heterogeneous region of wettability gradient surfaces [15].

The implications of this study for the development of so-called ‘easy wipe-off’ surfaces, as used in biomedical engineering [16], the design of non-toxic paints to control marine fouling on ship hulls [17] or in the prevention of biofilm formation and biodeterioration of monumental buildings are far reaching [18]. First of all, it emphasizes a currently voiced opinion that biofilm models distinguishing only a dense, base biofilm and a more open-structured surface biofilm [19] need to be extended with, by our definition, a linking film [20] (see also Fig. 3). The bacteria in

![Fig. 2. Average quantitative distribution of adhering streptococci on micro-patterned surfaces. The arrangement of the graphs is similar as in Fig. 1, with shaded areas indicating the hydrophobic lines. The standard deviation over six different locations, equally divided over two experiments, amounts 20%.](image)

![Fig. 3. Electron micrograph of a biofilm on a silicone rubber voice prosthesis with superimposed a biofilm model distinguishing a surface, base and linking film, in which the linking film bacteria are the initially adhering bacteria in direct contact with the substratum. Bar indicates 50 μm.](image)
the linking film are those that initially adhered to the substratum surface and whose retention under periods of high shear-off forces is under direct control of the substratum surface hydrophobicity and the distribution of heterogeneous hydrophilic sites. These initially adhering bacteria link the entire biofilm growing on top of them to the substratum and since their retention is governed by the substratum surface, so is the retention of the entire biofilm. Based on this study, it can be concluded that development of easy wipe-off surfaces for biofilm control in biomedical and industrial applications with fluctuating shear-off forces operative is feasible, but research efforts need to be re-directed from ‘bacterial adhesion’ towards ‘bacterial retention’.

References