Doctoral Thesis

Development and applications of optical tools to investigate the early steps of biofilm formation

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DEVELOPMENT AND APPLICATIONS OF OPTICAL TOOLS TO INVESTIGATE THE EARLY STEPS OF BIOFILM FORMATION

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH (Dr. sc. ETH Zurich)

presented by
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2014
Bacteria preferentially live as surface attached, matrix enclosed biofilms. In biofilms, bacteria show a considerably higher resistance against bactericides such as antibiotics and are less prone to the uptake by macrophages of the immune system. As biofilms bacteria colonize surfaces in medical settings including catheters, implants or heart valves, where they can cause severe and difficult to treat chronic infections. But also technical and industrial settings are affected. Biofilms form on the hull of ships or inside pipes where they cause corrosion and clogging, leading to tremendous costs.

To find novel ways to cope with these negative implications of biofilm formation, model systems are developed to investigate biofilms under laboratory conditions. For abstraction, these models are often reduced in complexity, featuring only parameters needed to answer a defined set of questions. However, in natural environments, bacterial communities are characterized by a variety of parameters including interbacterial communication, ambient fluid flow, matrix production, filamentation and heterogeneous adhesion to surfaces.

In this thesis, we developed model systems that include these environmental factors and ask how they co-regulate bacterial surface colonization. First, we asked how filamentation in bacteria impacts the colonization kinetics of heterogeneously adhesive surfaces. By a combined photolithography and lift-off process we created artificial surfaces to which bacteria adhered in well-defined patterns. We induced filamentation and found that it offers a bacterial community a kinetic advantage in colonizing heterogeneously adhesive surfaces over non-filamenting bacteria. This means that creating conditions which favor filamentation, including the administration of antibiotics, may have an adverse effect as it can accelerate the colonization of a surface instead of slowing it down.

Once a surface is colonized, the bacteria start to assemble into a biofilm. This is characterized by the formation of an enclosing matrix and an increase in the local cell density. A high population density favors cell to cell communication. One form of inter-bacterial communication is termed quorum sensing (QS), where bacteria produce and secrete small signaling molecules. The bacteria can detect a high concentration of the signaling molecule, which rises according to the population density and respond for instance with coordinated virulence factor expression or biofilm maturation. Ambient fluid flow, however, supposedly affects the local concentration of the signaling molecule,
Abstract

preventing QS in flow environments. Yet, bacteria are observed to QS in naturally occurring flow environments.

Thus, we secondly asked how QS is affected by ambient fluid flow and compared the onset of QS in early biofilms of *Pseudomonas aeruginosa*, which produces a prominent matrix, in static and flow conditions. The onset of QS and matrix formation was monitored by fluorescence based optical reporters with high temporal resolution. To investigate QS in the absence of matrix, we expressed the *P. aeruginosa* QS system recombinantly in *Escherichia coli*, which is not a strong matrix former and found that in the genetically engineered *E. coli* QS is prevented in flow conditions. This suggests that in hydrodynamic conditions, the biofilm matrix preserves the ability of bacteria to communicate via QS, whereas in static conditions QS is onset irrespective whether the matrix is present or not. This describes a so far overseen function of the biofilm matrix in flow environments and suggests that the QS signaling molecule accumulates in the matrix, preventing its washout by ambient flow.

Finally and in a collaborative effort, a new assay was developed for the in situ viability monitoring of surface attached bacteria. The assay applies non-toxic concentrations of propidium idodide (PI) in the culture medium to specifically stain dead bacteria. In combination, the entire bacterial population expresses the green fluorescent protein (GFP). GFP replaces the commercially available and often used stain SYTO® 9, which negatively influences bacterial growth. By replacing SYTO® 9 with GFP the assay is suitable for optical real-time monitoring of bacterial viability. Using *E. coli* as a model organism, the viability of bacteria on a bioactive surface that kills bacteria upon contact was monitored by fluorescence microscopy. We provide an assay itself not interfering with bacterial viability, capable of in-situ viability monitoring with high temporal resolution to characterize engineered surfaces in physiological relevant conditions.

Including environmental factors such as filamentation triggers, ambient flow, biofilm matrix, heterogeneity of surfaces and interbacterial communication into model systems helps to better understand the bacterial physiology and to find treatments to reduce the negative impacts bacterial biofilms can have.
Zusammenfassung


In dieser Arbeit wurden Modellsysteme entwickelt, die diese äusseren Faktoren berücksichtigen, und untersucht, wie diese die bakterielle Oberflächenbesiedlung mitregulieren. Als erstes wurde untersucht, wie die Filamentierung bei Bakterien die Besiedlungskinetik von ungleichmässig adhäsviven Oberflächen beeinflusst. Mit einem kombinierten Fotolithografie- und lift-off Prozess wurden künstliche Oberflächen hergestellt, an die Bakterien in genau definierten Mustern anhaften können. Die Filamentierung wurde eingeleitet und es wurde gezeigt, dass diese, im Vergleich mit nicht-filamentierenden Bakterien, der bakteriellen Gemeinschaft einen kinetischen Vorteil bei der Besiedlung heterogen adhäsviver Oberflächen verschafft. Dies bedeutet, dass die Begünstigung der Filamentierung, beispielsweise durch die Anwendung von Antibiotika, nachteilige Effekte haben kann, da dies die Besiedlung einer Oberfläche zu beschleunigen vermag, anstatt sie zu verlangsamen.
Zusammenfassung


Deshalb wurde als zweites untersucht, inwiefern Quorum sensing durch Umgebungsfluss beeinträchtigt wird. Dabei wurde das Einsetzen von Quorum sensing in frühen Biofilmen von Pseudomonas aeruginosa, die eine prominente Matrix ausbilden, sowohl in statischen als auch in Fliessumgebungen verglichen. Das Einsetzen des Quorum sensings und der Matrixproduktion wurde vermöge fluoreszierender optischer Reporter mit hoher zeitlicher Auflösung überwacht. Um Quorum sensing in Abwesenheit einer Matrix zu untersuchen, wurde das Quorum sensing-System von P. aeruginosa rekombinant in Escherichia coli, die keine ausgeprägte Matrix ausbilden, exprimiert und dabei gezeigt, dass in den gentechnisch veränderten E. coli Quorum sensing in Fliessumgebungen nicht stattfindet. Unsere Resultate legen nahe, dass die Biofilmmatrix in hydrodynamischen Umgebungen die Kommunikation mittels Quorum sensing ermöglicht, wohingegen in statischen Umgebungen Quorum sensing einsetzt, unabhängig davon, ob eine Matrix vorhanden ist oder nicht. Dies beschreibt eine bisher unerkannte Funktion der Biofilmmatrix in Fliessumgebungen und legt nahe, dass sich die Quorum sensing Signalmoleküle in der Matrix ansammeln können und diese deren Auswaschen durch den umgebenden Fluss verhindert.

E. coli als Modellorganismus wurde hier das Abtöten von Bakterien auf einer bioaktiven Oberfläche durch Fluoreszenzmikroskopie aufgezeichnet. Es wurde ferner gezeigt, dass die vorgestellte Methode ihrerseits keine Toxizität gegenüber den Bakterien aufweist und in der Lage ist, in physiologisch relevanten Bedingungen das Abtöten durch die Oberfläche in situ mit hoher zeitlicher Auflösung festzuhalten.

Das Einbeziehen von Umgebungsfaktoren, so wie Auslöser zur Filamentierung, Umgebungsfluss, Biofilmmatrix, Heterogenität von Oberflächen und interbakterielle Kommunikation in Modellsysteme hilft, die bakterielle Physiologie besser zu verstehen und neue Verfahren zu finden, um die negativen Auswirkungen, die bakterielle Biofilme haben können, zu reduzieren.
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>C12-HSL</td>
<td>3-oxo-C_{12}-homoserine lactone</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>conA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CTC</td>
<td>5-cyano-2,3-ditolyl tetrazolium chloride</td>
</tr>
<tr>
<td>DMOAC</td>
<td>dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>eDNA</td>
<td>extracellular DNA</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced GFP</td>
</tr>
<tr>
<td>EM-CCD</td>
<td>electron multiplying charge-coupled device</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substances</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HSL</td>
<td>homoserine lactone</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>MAPL</td>
<td>molecular assembly patterning by lift-off</td>
</tr>
<tr>
<td>NMP</td>
<td>N-Methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
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</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>polydimethylsiloxane</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-L-lysine</td>
</tr>
<tr>
<td>PLL-g-PEG</td>
<td>poly(L-lysine)-g-poly(ethylene glycol)</td>
</tr>
<tr>
<td>PMOXA</td>
<td>poly(2-methyl-2-oxazoline)</td>
</tr>
<tr>
<td>QS</td>
<td>quorum sensing</td>
</tr>
<tr>
<td>QSR</td>
<td>quorum sensing reporter</td>
</tr>
<tr>
<td>RNaseB</td>
<td>Ribonuclease B</td>
</tr>
<tr>
<td>VASE</td>
<td>variable-angle spectroscopic ellipsometry</td>
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1 Scope of the Thesis

1.1 Motivation

At the end of the 19th century, medical microbiology encountered a leap with the understanding that microorganisms are responsible for a number of infectious diseases. The Koch’s postulates, named after the famous microbiologist Robert Koch (1843-1910), were dominating medical microbiology for the better part of the upcoming century. These four principles identify a microorganism as causative agent for a given disease by (i) detection of the microorganism in all diseased but not in healthy individuals, (ii) isolation and cultivation of the microorganism in pure culture, (iii) reconstitution of the disease in healthy organisms by the pure culture and (iv) re-isolation of the microorganism from the inoculated host. It was the second postulate, the demand for growth as pure isolated culture, that dictated the microbiological techniques of the following decades. Pure bacterial batch cultures were thus subsequently used to detect microbicidal and the observed effectivity of antibiotics against batch cultured bacteria led to a proper euphoria. In the 60s and 70s of the last century, it was thought that infectious diseases were defeated. Today we know that this was terribly wrong.

In the mid 20th century the awareness arose that in natural and technical habitats microorganisms typically grow as surface attached slime-enclosed communities rather than as planktonic individuals. These so called biofilms are ubiquitous in nature, medicine and industry and today considered to be the predominant life style of bacteria. Living as a biofilm, bacteria show considerable resistance against antimicrobial agents and are tough to remove. It became evident that biofilms play an important role in medicine as source of difficult to treat chronic infections and in technical settings such as the hull of ships or inside pipes, where they cause corrosion and clogging. To this day the implications of biofilms are underestimated which is remarkable given that biofilm research has dramatically increased in the last 30 years.

The increasing development of resistant pathogens and the observation that certain abiotic agents are ineffective in chronic diseases demand the development of model systems that reflect the natural habitats of microbes more accurately and allow to find and test agents with enhanced efficiency against biofilms. Such model systems should include bacterial attachment to surfaces, bacterial morphology and biofilm formation. Since most natural habitats en-
counter flow, model systems should include hydrodynamic environments. A high temporal resolution allows to acquire kinetic data of how antimicrobial compounds act. Non-invasive staining and imaging techniques allow for in situ real time measurements rather than the snapshot of end point experiments.

The tools available to conduct research on biofilms have increased and improved over the last decades. High resolution imaging and fluorescence labeling as well as highly sensitive detectors allow to retrieve information how processes work on a single cell level. In contrast to batch cultures, a high temporal resolution can be achieved with modern and fully automated microscopic systems which is well suited for the study of single bacteria, revealing more precisely when processes such as cell death, communication or gene expression start, computers allow to simulate situations difficult to implement experimentally and inconceivable amounts of data can be processed.

With advanced techniques and updated models research aims to gain detailed insight on how bacteria colonize surfaces, establish in their environment and are killed by biocides. This is the base for the development of a new generation of drugs and may help to step forward in the treatment of infectious diseases.

1.2 Objectives

To gain insight into the early steps of biofilm formation, the first stages of this process are addressed in this thesis by development and application of optical reporter tools to investigate the transition of individual bacteria to bacterial communities. Three specific aims are outlined, for each of which a short summary of the questions and results is given.

1.2.1 A tool for online monitoring of bacterial viability upon initial attachment to bioactive surfaces (chapter 3)

The initial attachment of bacteria to substrates is a crucial step in biofilm formation and offers a first possibility to prevent bacterial surface colonization. In this chapter, we present an improved optical tool to characterize the efficiency of antimicrobial surface coatings by monitoring the viability of attached single bacteria. In contrast to existing assays, our approach is non-invasive, thus eliminating false positives, and in real time, allowing to derive a kinetic of the bioactive compound’s mode of action. The content of this chapter has been published in *Biointerphases* August 2013, 8:22 by I. Avalos Vizcarra, P. Emge, P. Miermeister, M. Chabria, R. Konradi, V. Vogel and J. Möller. P. Emge contributed to this work as part of a collaboration.
1.2 Objectives

1.2.2 Effect of bacterial filamentation on the colonization of engineered surfaces decorated with adhesive sites within a biopassive layer (chapter 4)

The ability of bacteria to grow into high aspect ratio filaments has been suggested as a strategy by which bacteria can escape from host immune defense effectors or macrophage uptake and to provide temporary tolerance towards certain antibiotics. In this chapter, we demonstrate that bacterial filamentation accelerates the colonization of heterogeneously adhesive surfaces compared to non-filamentous bacteria. Filamentous bacteria bridge non-adhesive areas between adhesive sites significantly faster than non-filamentous bacteria and thus promote surface colonization. A mathematical model reproduced the experimental findings and allowed for extrapolation to conditions that were not experimentally addressed. Long regarded as a detrimental effect of stress response, filamentation has shown to be advantageous in many situations. In this work we discover an additional advantage of filamentation to bacteria in the colonization of heterogeneous surfaces. The content of this chapter has been published in New Journal of Physics 15 (2013) 125016 by P. Emge, J. Möller, I. Avalos Vizcarra, P. Kollmannsberger, and V. Vogel.

1.2.3 Role of the *P. aeruginosa* extracellular polymeric substances (EPS) matrix in quorum sensing under fluid flow (chapter 5)

Bacteria often grow as matrix-enclosed biofilms on technical and industrial settings where they cause corrosion and clogging and on implants, catheters or tissue in the human body, where they cause chronic and difficult to treat infections. Quorum sensing (QS), an interbacterial communication by small molecules, plays an important role in the development of virulence, but is influenced by the hydrodynamic environment of the biofilm’s habitat. In this paper, we show that in hydrodynamic environments, the biofilm matrix is required for QS in *Pseudomonas aeruginosa*, an opportunistic human pathogen. This implies that the biofilm matrix retains QS signaling molecules and is needed for QS regulated virulence development in flow environments. Interfering with this retention effect of the biofilm matrix may be a promising treatment against biofilm caused diseases.
2 Introduction

The preference of bacteria to grow as surface attached biofilms comprises three major steps, (i) the initial adhesion of planktonic bacteria to a substratum, (ii) the growth and further colonization of the surface (monolayer formation) and (iii) the formation of a biofilm, typically associated with the formation of a matrix consisting of extracellular polymeric substances (EPS) such as proteins, oligosaccharides and DNA. The formation of a biofilm is usually divided in further steps of maturation and again dispersal of planktonic cells [1]. Here, we focus on the three early steps of bacterial surface association and discuss representative samples further.

2.1 Initial adhesion

Binding of bacteria to a surface or tissue is the initial and first critical step to subsequent formation of a biofilm. If the surface is an implant, indwelling device or heart valve, this can lead to chronic infections which are difficult to treat. On the hull of ships or inside pipes biofilms can promote corrosion and clogging and thus cause tremendous economical costs.

One strategy to prevent biofilm formation on engineered surfaces is to interfere with the initial adhesion of bacteria. For this, antimicrobial surface coatings that prevent initial adhesion by killing bacteria upon contact or leaching of a bioactive compound have been developed. Biopassive surface coatings prevent the contact of bacteria with the surface by means of steric hindering. Long polymers such as poly(ethylene glycol) (PEG) or poly(2-methyl-2-oxazoline) (PMOXA) are grafted onto a substrate via poly-L-lysine (PLL). These brush-like structures self-assemble into monolayers on negatively charged surfaces and reliably prevent bacterial adhesion [2]. For active surfaces, biocides, often quaternary ammonium compounds, but also antibiotics, are covalently linked to the PEG or PMOXA coatings which results in killing bacteria upon contact [3] and thus prevent the surface colonization and biofilm formation. Typically, bacterial adhesion is a consequence of protein adhesion to a surface and many analytical tools to characterize functionalized surfaces measure the protein adlayer thickness [4]. Microscopic techniques allow us to assess the adhesive properties of a surface but give no information on the biocidal activity of a bioactive compound. Since even a strong reduction of initial bacterial adhesion eventually results in colonization of the surface...
Introduction

Figure 2.1: Peyer’s patches in the mammalian intestine\textsuperscript{1} are a preferred site for bacterial adhesion.

[3], the kinetic of a bioactive compound is of great interest. Commercially available life/dead stainings exist, but these usually do not allow to derive a kinetic of killing in real time or they themselves are toxic to the bacteria and lead to false positives. An ideal tool to assess the effect of bioactive compounds on surface allows for real-time in situ monitoring of bacterial viability on a surface.

2.2 Surface colonization

The process of surface coating may be imperfect and coatings can degrade and corrode over time [5], leaving surfaces with defects to which bacteria can adhere. From these nucleation points, single bacteria start to divide and form microcolonies, connect to each other by overcoming the non-adhesive areas and form a monolayer. Not only engineered surfaces but also living tissues, for example Peyer’s patches the intestinal epithelium, exhibit sites where bacteria preferably adhere [6] (Figure 2.1). Thus, bacteria approaching surfaces encounter heterogeneity and most likely adhere in patterns. Morphological diversity, especially high aspect ratios, can be of advantage at that stage of surface colonization as long filamentous bacteria overcome the distance between adhesive spots easier and contribute to a fast monolayer formation [7]. Filamentation occurs if the bacterial growth continues but no septation occurs [8]. Bacterial division requires the assembly of the complete divisome, a complex of over 10 proteins that assemble mid-cell during the division [9]. The individual proteins of the divisome are assembled in an ordered process to ensure that the cell wall degradation needed for daughter cell separation does not take place before all the enzymes needed for the re-assembly of the

\textsuperscript{1}Source of the intestine image: https://www.sccollege.edu/StudentServices/HealthWellnessCenter/AlcoholEffects/Pages/intestines.aspx
cell wall are present [10]. As part of the divisome, FtsI (formerly PBP3) is a transpeptidase required for cross-linking septal peptidoglycans in the division process. Blocking FtsI prevents the constriction of the mid-cell Z-ring in bacterial division and results in the formation of filaments with multiple nucleoids [11]. Inactivation of FtsI and thus filamentation can be triggered by conditional environments such as temperature, pH, pressure or some antibiotics of the $\beta$-lactam class [12]. When exposed to $\beta$-lactam antibiotics, the suppressed septation during filamentation helps the bacteria to temporarily limit the effect of the antibiotic [12]. Further, the high aspect ratio of bacterial filaments considerably slows down their uptake by macrophages [13], therefore providing an escape mechanism for innate immunity. Filamentation thus may help to evade unfavorable conditions and to rapidly build a monolayer even on antifouling surfaces.

2.3 Early biofilm formation

2.3.1 Biofilms

After adhering and colonizing a surface, bacteria start to form into a biofilm in a multistep process. This usually involves production of extracellular polymeric substances (EPS) with the properties of a slime and phenotypical differentiation of cells within the community. Eventually single bacteria are released from the biofilm again as planktonic cells that can colonize new habitats [1]. This last step closes the cycle of the bacterial life style (Figure 2.2). Although both planktonic and sessile bacteria occur in nature, it is estimated that 99% of the microbial biomass exists as biofilms [15]. Biofilms are found in a wide
range of habitats including aquatic environments, hot springs, deep-sea vents, soils and animal tissue [16, 17]. Biofilms are also of great clinical interest, as they can inhabit medical devices like catheters, stents prosthetic heart valves and implants where they account for the majority of chronic infections [18–20]. Bacteria in the biofilm mode of life show enhanced resistance to antibiotics, disinfecting agents and phagocytes [18, 19, 21, 22] and it offers protection from toxic agents, dehydration and UV irradiation [18].

2.3.2 Quorum sensing

In biofilms, bacteria are spatially organized and typically found at a higher population density than their planktonic counterparts [20, 23]. The close proximity of neighboring bacteria influences intercellular interactions [23] and provides an optimal environment for cell-to-cell signaling. Quorum sensing (QS), as one form of interbacterial communication, is established by the bacterial synthesis, secretion and detection of small inducer molecules. Various QS systems exist [24], but the basic principle remains the same. The best studied QS systems are the LuxI/R-type systems found in gram-negative bacteria, such as *Vibrio fischeri* and *Pseudomonas aeruginosa*. LuxI-type synthases catalyze the production of acyl-homoserine lactone (HSL) molecules that are released by the bacteria. Within the local microenvironment, the HSL molecules accumulate according to the bacterial population density. Once a threshold concentration is reached, the HSL molecules bind to a cognate intracellular LuxR-type transcription regulator that in turn promotes target gene expression (Figure 2.3). The Substrate of LuxI-type HSL synthase is S-adenosyl-L-methionine (SAM) and acyl-acyl carrier protein (acyl-ACP) [25]. Both SAM and acyl-ACP are key compounds in the bacterial metabolism and it was demonstrated that recombinantly expressed LuxI-type proteins in bacteria not using HSL based QS results in the production of HSLs [26].

Commonly, QS is described as a mechanism of gene regulation solely dependent on population density but it revealed to be part of a complex system of regulation and superregulation [27]. In addition to population density, other parameters affect the inducer molecule concentration, including the nature of the local environment, and modulate the onset of QS [28]. This led to the definitions of the terms “diffusion sensing” and “efficiency sensing” [29, 30] which take in account further parameters that affect the inducer molecule concentration, such as advection, diffusion or degradation. The actual “quorum”, i.e. the number of bacteria that are needed to establish a QS dependent gene expression, is in a major way influenced by the environmental condition of a bacterial population. The inducer concentration depends on the production and degradation rate, the diffusion properties and the local hydrodynamic conditions [31].
2.3 Early biofilm formation

If no bulk liquid is present within or around bacterial colonies, as found in e.g. dry soil or colonies on agar plates, the diffusion of the inducer molecule is very limited and accumulation happens fast. However, most bacterial habitats are surrounded by aqueous solutions and QS systems were traditionally studied in liquid batch cultures [32]. Within liquid cultures, the inducer molecules freely diffuse into the bulk fluid [32] and thus the inducer concentration near the bacteria is decreased. However, a batch culture represents a closed system with a limited volume of medium, and the HSL concentration within the system will increase over time, unless the molecules are hydrolytically degraded [31]. Most bacterial communities, however, are situated in open environmental systems, where a permanent exchange of the superfluent medium occurs. Depending on the mass transport of the overlays liquid, the constant medium exchange will adversely affect the accumulation of signaling molecules as they may be washed away [31, 33]. So far, only few studies systematically investigate the effect of open flow systems on the accumulation of the inducer molecules [32, 34] and the flow conditions that prevent the onset of QS remain unknown.

**P. aeruginosa quorum sensing**  In the last decades, *P. aeruginosa* has become a paradigm organism for the study of quorum sensing [35, 36]. Two hierarchically arranged quorum sensing systems are described in *P. aeruginosa*, LasI/R and RhlI/R. LasI is a synthase producing 3-oxo-C12-homoserine lactone (C12-HSL, Figure 2.4) which, above threshold concentration, binds its cognate receptor LasR. The LasR-C12-HSL complex activates a variety of genes including the second QS system RhlI/R by activating RhlI and RhlR expression [37]. The RhlI/R system produces and responds to N-butyryl-HSL (C4-HSL, Figure 2.4) which regulates another set of genes. QS regulatory networks typically control several percent of a bacterial genome. Some of these
genes may be additionally controlled by a third factor or alternative, QS independent ways of upregulation are available if the QS system is shut down. In *P. aeruginosa* for example many of the QS regulated genes are affected by the stationary phase sigma factor RpoS [27] and are not expressed before stationary phase, irrespective of inducer or receptor levels [38–40]. The presence of exogenous HSL throughout the growth of signal generation mutants does not alter the timing of most QS controlled genes in *P. aeruginosa*, which suggests additional regulators involved in QS [41, 42]. The majority of genes that require additional regulators are controlled by LasI/R promoters whereas *rhl*-promoters generally seem to be directly affected by receptor and inducer levels and do not need further factors [27].

**E. coli quorum sensing**  *E. coli* lacks a LuxI homologue and therefore does not synthesize homoserine lactone (HSL) molecules [43, 44]. It does, however, have a LuxR homologue, SdiA, which detects HSL’s produced and released by other species. SidA controls the upregulation of acid fitness and downregulation of flagella and the locus of enterocyte effacement (LEE) in enterohemoragric *E. coli* [45, 46] and is involved in the process of cell division [47].

*E. coli* employs another QS system based on the autoinducer 2 (AI-2) which is a furanosyl borate diester that serves as a common inducer molecule in many gram negative and gram positive species [48, 49]. The AI-2 based QS system allows communication across species and regulates chemotaxis, motility and biofilm formation [49].
Another QS system employing a third autoinducer molecule, AI-3, has been reported in *E. coli*. This system can be activated by epinephrin and norepinephrin, thus allowing inter-kingdom communication of *E. coli* with its host mammalian cell [50].

### 2.3.3 Biofilm Matrix

While a bacterial biofilm itself consists to 98% of water, the highly structured architecture of bacterial biofilms is commonly supported by an extensive matrix consisting of polysaccharides, proteins and DNA (extracellular polymeric substances, EPS) [51]. Within the biofilm, the EPS establishes a network of channels that provides bacteria deep within a biofilm with a constant nutrient supply and allows removal of metabolic products [52, 53]. The specific composition of the matrix varies substantially between bacterial species and residing bacteria can modify the matrix during their life cycle by degradation, synthesis and secretion of matrix compounds [54]. Even biofilms of the same species can differ in their EPS, depending on the environmental conditions such as shear force, nutrients availability or temperature [33, 55], which makes it difficult to assign a universal composition, function or structure to a matrix. Generally, a matrix provides mechanical stability, protection from chemical and mechanical stress, biocides or grazing predators and the host immune defense [18].

The hydrophobic and negatively charged matrix polymers can bind cations and positively charged amino acid residues of enzymes and serve as an external enzyme system and sink for small molecules [51]. The EPS thus significantly reduces and retards the diffusion of small molecules, which might explain some extent the enhanced tolerance against antimicrobial agents [20, 56–58]. In addition, a substantial difference in signaling molecule concentration within a *P. aeruginosa* biofilm grown in open flow cell systems compared to the superfluent medium was reported [59].

Retarded diffusion and immobilization of small compounds within the EPS may thus influence the local concentration of the QS signaling molecules. If the EPS does influence the inducer accumulation, the kinetics of matrix production should correlate with the QS onset. However, no data is available for the kinetics and the timepoint at which matrix production starts.

The diffusion of small molecules through a biofilm is an important characteristic of a matrix, as it contributes to a biofilms enhanced tolerance towards biocides. A biofilm matrix is a complex hydrogel consisting to 98% of water. 99.8% of this water has a diffusion coefficient 10% lower than that of bulk water [51]. Diffusion coefficients of various compounds were measured and show a large spectrum, depending on the biofilm investigated and the measurement method [60]. The penetration of antibiotics through biofilm matrices is limited.
P. aeruginosa matrix  The EPS of different *P. aeruginosa* strains consist of different polysaccharides. The matrix of mucoid strains found in the lung of cystic fibrosis patients typically contain alginate as the major polysaccharide whereas it is absent in strains PA14 and PA01 [64]. Alginate is the best characterized polysaccharide of the *P. aeruginosa* EPS and is composed of manuronic acids and guluronic acids [63]. The dominating polysaccharide in strain PA14 is Pel, which is reported to be glucose rich [65, 66], but its exact structure is yet unknown. PAO1 finally expresses mainly the Psl sugars as matrix components [67]. The Psl exopolysaccharide is a pentameric repeat of mannose, rhamnose and glucose, whereas mannose is the dominating component (Figure 2.5) [68]. The diversity in the polysaccharide composition and structure allows for specific staining. Several fluorescently labeled lectins were used to stain the different polysaccharides of different strains of *P. aeruginosa* [69, 70]. One of these lectins, concanavalin A (conA), preferentially binds α-D-mannopyranosyl [71], a sugar part of the Psl polysaccharide (Figure 2.5).

Apart from polysaccharides, the matrix contains various proteins, most of them hydrolases [72]. In *P. aeruginosa*, many matrix proteins were recently reported to be associated with outer membrane vesicles (OMVs) [73], which are secreted by the bacteria and have important biological functions [74]. The amount of protein in the matrix exceeds the polysaccharides by mass [52], but their prevailing role is likely a functional one rather than a structural one.

The role of extracellular DNA (eDNA) as a matrix component has long been underestimated. DNA exits the cells by active secretion or controlled lysis [61]. *P. aeruginosa* biofilms treated with DNAses disperse which demonstrates the structural role of eDNA. eDNA can also act as adhesive substance and change
the hydrophobic properties of a biofilm matrix [61]. In *P. aeruginosa* biofilms, eDNA is identical to genomic DNA [75] and seems to reach the matrix mainly by cell lysis [76]. Most recent work showed that the anionic eDNA can bind some cationic antibiotics and thus protect the resident bacteria and contribute to the enhanced resistance of biofilms against antibiotics [77].

Taken together, the matrix of *P. aeruginosa* seems to be a complex, versatile and dynamic construct with different functions of which not all have been elucidated yet. It should also be considered that the role of different matrix components changes with the different stages of biofilm development. Furthermore, the matrix is influenced by the environmental conditions such as nutrients, shear forces or temperature and it can be modified by the resident bacteria themselves [51].

**E. coli matrix**  Most K-12 derivatives of the laboratory strain *E. coli* do not form prominent biofilms. However, the presence of a functional F pilus restores the ability of these strains to form thick biofilms [78].

*E. coli* hardly adheres to plain glass surfaces but with a colonizing partner such as *P. aeruginosa* glass substrates can be colonized [79]. Alternatively, the glass surface can be mannosylated, for example by coating the glass substrate with a mannosylated protein. *E. coli* then adheres specifically to the presented mannose residutes by its type 1 pili via the tip adhesin FimH [7].

The *E. coli* matrix sugars are poly-β-1,6-N-acetyl-glucosamine (PGA), colanic acid and cellulose. PGA is involved in adhesion to substrates and between cells [80], cellulose is involved in the biofilm formation at the air-liquid interface and colanic acid, which usually is a component of the bacterial capsule, can be found in significant amounts in the *E. coli* matrix [78] where it is involved in the maturation of biofilms [81].
References


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References


3 Fluorescence-based in situ assay to probe the viability and growth kinetics of surface-adhering and suspended recombinant bacteria

This chapter was published in Biointerphases August 2013, 8:22 under the terms of the Creative Commons Attribution 2.0 license. P. Emge contributed to this work as part of a collaboration. I. Avalos Vizcarra, J. Möller, M. Cabria, R. Konradi and V. Vogel designed research, P. Emge cloned the pHis-eGFP plasmid, P. Miermeister developed semiautomatic image analysis software, I. Avalos Vizcarra and J. Möller performed research, I. Avalos Vizcarra, P. Emge, P. Miermeister and J. Möller analyzed data (P. Emge analyzed the data shown in Figure 3.4, prepared the graphs, images and caption; P. Emge designed research, performed experiments and analyzed data of Figure S 3.7 and prepared the graph and caption). I. Avalos Vizcarra, P. Emge, R. Konradi, V. Vogel, and J. Möller wrote the paper (P. Emge wrote parts of the Methods, Results and Discussion). All authors read and approved the final manuscript.

3.1 Abstract

Bacterial adhesion and biofilm growth can cause severe biomaterial-related infections and failure of medical implants. To assess the antifouling properties of engineered coatings, advanced approaches are needed for in situ monitoring of bacterial viability and growth kinetics as the bacteria colonize a surface. Here, we present an optimized protocol for optical real-time quantification of bacterial viability. To stain living bacteria, we replaced the commonly used fluorescent dye SYTO® 9 with endogenously expressed eGFP, as SYTO® 9 inhibited bacterial growth. With the addition of nontoxic concentrations of propidium iodide (PI) to the culture medium, the fraction of live and dead bacteria could be continuously monitored by fluorescence microscopy as demonstrated here using GFP expressing Escherichia coli as model organism. The viability of bacteria was thereby monitored on untreated and bioactive
dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride (DMOAC)-coated glass substrates over several hours. Pre-adsorption of the antimicrobial surfaces with serum proteins, which mimics typical protein adsorption to biomaterial surfaces upon contact with host body fluids, completely blocked the antimicrobial activity of the DMOAC surfaces as we observed the recovery of bacterial growth. Hence, this optimized eGFP/PI viability assay provides a protocol for unperturbed in situ monitoring of bacterial viability and colonization on engineered biomaterial surfaces with single-bacteria sensitivity under physiologically relevant conditions.

3.2 Background

Clinically relevant nosocomial infections are frequently caused by adherent bacteria and the subsequent biofilm formation within tissues or on biomaterial surfaces [1]. Surface biofouling commonly starts with the adhesion of individual bacteria that subsequently grow into mature biofilms. To prevent bacterial adhesion and growth already during the pre-biofilm phase, two main surface engineering strategies have evolved so far: the development of non-fouling “stealth” surface coatings that inhibit adhesion of proteins and bacteria [2–4] and the engineering of bioactive materials, which upon bacterial contact or release of the active molecules interfere with bacterial viability [5–10]. To compare the antimicrobial properties of surface coatings and to study the kinetics of bacterial surface colonization, assays are needed that allow for in situ monitoring of bacterial adhesion and viability. The gold standard for bacterial viability tests has long been quantification of colony forming units (CFU) by plating bacterial suspensions that were incubated with the test surface on nutrient agar [11]. Counting bacterial colonies, which result from plating suspended viable and cultivatable bacteria, however, does not account for the inherent phenotypic heterogeneity and the ability of the bacteria to persist in dormant states [12, 13]. Furthermore, plating assays lack the ability to measure the colonization and viability kinetics directly on the test surface and might not be representative for the surface-attached bacterial population.

An alternative to determine bacterial viability is to probe for the bacterial membrane integrity that is maintained by energy-dependent processes in living bacteria and is lost upon bacterial death [14]. Membrane integrity can be tested optically by using a combination of membrane permeable and impermeable fluorescent dyes that selectively enter live and dead bacteria (Figure 3.1a) [8, 14–17]. While being broadly employed as endpoint staining assays to determine the viability of single bacteria and bacterial colonies directly on the test surface, these assays are not optimized for real-time in situ bacterial viability monitoring. Particularly when DNA intercalating dyes like SYTO® 9 and
3.2 Background

Figure 3.1: Bacterial viability assays in comparison. (a) Conventional endpoint dual staining bacterial viability assay and (b) eGFP/PI assay optimized for in situ bacterial viability monitoring. The dual staining assay commonly employs two DNA stains, SYTO® 9 (green triangle) and propidium iodide (PI, red rectangle) that both intercalate into the bacterial DNA. SYTO® 9 diffuses passively into living bacteria whereas PI cannot pass intact bacterial membranes and only enters permeabilized dead bacteria. The use of constitutive eGFP expression rather than SYTO® 9 provides direct detection of viable bacteria without the addition of a fluorescent dye and circumvents the competitive displacement of SYTO® 9 by PI that can result in a dual staining of dead bacteria.

propidium iodide (PI) are used [18], the impact of the potentially toxic stains on bacterial physiology has to be considered to avoid false negative results [19]. Furthermore, since both stains target DNA, the competitive displacement of the SYTO® 9 (live stain) by the high affinity PI (dead stain) upon membrane breakdown can affect the staining reliability [20]. To eliminate the competitive displacement of the two DNA stains and the demand for prolonged incubation times caused by the passive diffusion of the SYTO® 9 live stain through the bacterial membrane, it was suggested to replace SYTO® 9 with green fluorescent protein (GFP) expressed by the bacteria as demonstrated previously for flow cytometry applications (Figure 3.1 b) [21]. Although flow cytometry has been used to measure the viability of GFP expressing bacteria adsorbed to polystyrene beads functionalized with antimicrobial coatings [22], it cannot be applied for continuous in situ bacterial viability monitoring on planar surfaces.

Building upon those observations, we present an optimized protocol to probe the viability and growth kinetics of surface-adhering and suspended bacteria using non-toxic concentrations of propidium iodide and *Escherichia coli* that express the fluorescent protein GFP. Beyond calibrating the assay and monitoring *E. coli* surface colonization kinetics on bare glass substrates, we demonstrate that this assay is applicable to monitor the inactivation kinetics of *E. coli* in contact with antimicrobial surface coatings, using dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride (DMOAC) coated glass surfaces as model substrate. To kill bacteria, the quaternary ammonium chloride com-
plexes of surface-bound DMOAC have to directly interact with the bacterial membrane [23]. We previously showed that bacterial fimbriae strongly influence the unspecific adhesion of E. coli to engineered surfaces [4]. Type 1 fimbriae (7 nm diameter, several 100 µm length) protrude from the bacterial membrane thereby preventing the bulk bacterial body from direct interaction with the underlying material surface. To ensure a physical contact of the bacterial membrane with the material surface, we used here the non-fimbriated K-12 derivative AAEC191A E. coli strain. In addition, we highlight the effect of serum protein adsorption on the bactericidal properties of antimicrobial surfaces. We incubated the DMOAC surfaces with fetal bovine serum (FBS) to mimic the physiological situation where serum proteins adsorb to engineered biomaterials upon contact with host body fluids.

3.3 Methods

3.3.1 Bacteria

Non-fimbriated E. coli AAEC191A bacteria, a derivative of E. coli K-12 MG 1655 containing a deletion in the entire fim-cluster [24] was provided by Prof. E. Sokurenko, University of Washington, Seattle, USA. For GFP expression, chemocompetent AAEC191A E. coli were transformed with eGFP pHis plasmid under the control of the tac-promoter (AAEC191A pHis-GFP). To obtain E. coli that express eGFP under the control of the constitutive rpsm-promoter (AAEC191A rpsm-GFP), E. coli AAEC191A were transformed with the rpsm-GFP plasmid that was extracted from the original fusion library strain MG1655 rpsm-GFP [25] by Qiaprep Spin Miniprep kit (Qiagen 27106). Transformed bacteria were selected by cultivation on LB agar plates supplemented with either 100 µg/mL ampicillin (pHis-GFP) or 50 µg/mL kanamycin (rpsm-GFP). Bacterial precultures were inoculated from glycerol stocks into LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) containing appropriate antibiotics. To induce GFP expression in AAEC191A pHis-GFP E. coli, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Applichem A1008) was added. LB precultures were grown overnight at 37 °C under continuous shaking at 180 rpm (Infors Unitron HT). To ensure defined culture conditions for the bacterial growth and viability assays, bacteria from the overnight culture were centrifuged at 1700 g, washed three times and subcultured in 20 mL minimal M9 medium (1x M9 salts (Sigma-Aldrich M6030), 10 mM MgSO4 (Sigma-Aldrich 63126), 10 g/L glucose (Sigma-Aldrich G8270), 0.5 mM CaCl2 (Sigma-Aldrich C5080), 1x MEM vitamins (Gibco 11120), 1x MEM amino acids (Gibco 11130)) supplemented with the appropriate antibiotics and 0.1 mM IPTG for AAEC191A pHis-GFP E. coli. Bacteria were subcultured at 37 °C, 180 rpm until exponential growth phase (OD600 = 0.3-0.8).
Bacteria were harvested by centrifugation at 1700 g followed by three washing steps. Immediately before the experiment, bacteria were resuspended in M9 medium that contained the strain-specific antibiotics, IPTG as well as 0, 3, 30 µM PI (Sigma-Aldrich, 81845) and 6 µM SYTO® 9 (Invitrogen L13152), respectively.

### 3.3.2 Growth curve measurements

Growth curves of suspended bacteria were recorded by turbidity measurements at 600 nm in 96 well plates (Tecan Infinity 200 Pro plate reader). Kinetic measurements were performed every 15 minutes at 37 °C and continuous shaking. Bacteria were inoculated to an initial turbidity of 0.01 at 600 nm in M9 medium containing appropriate antibiotics, PI and SYTO® 9.

### 3.3.3 Viability assay

For kinetic viability measurements under physiological conditions, bacteria were cultivated in an ibidi® glass bottom flow chamber (ibidi, 80168) within a temperature-controlled microscope incubator to guarantee constant nutrient supply and optimal growth conditions at 37 °C. Bioactive surfaces were prepared according to published work [23]. Briefly, glass cover slides, that later resemble the bottom slide of the flow chamber, were exposed to air plasma for 15 seconds (Harrick Plasma, PDC-32G) followed by dipping into a 5% (v/v) aqueous DMOAC (Sigma-Aldrich) solution for 1 second, and drying at 37 °C overnight. To test the effect of protein pre-incubation on the antimicrobial activity of the DMOAC coatings, slides were incubated in undiluted fetal bovine serum (FBS, Thermo Scientific SH30071.02) for 1 h prior to assembly of the flow chamber. Bare glass cover slides were attached to the ibidi® chambers as control surfaces. The bacterial suspension (OD600 0.05) in M9 medium containing different concentrations of PI and SYTO® 9, was directly added to the flow chamber and immediately transferred to an epifluorescence microscope (Nikon TE2000-E) for in situ viability monitoring. Adhesion of bacteria to the glass bottom slide was allowed for 5 minutes before the flow chamber was gently washed with 5 mL M9 medium (flow rate 0.01 mL/min to remove non-adherent bacteria. As control staining at defined time points, the BacLight™ viability kit (Invitrogen, L13152) was used according to the supplier instructions.

### 3.3.4 Ellipsometry

The adsorbed dry film thickness of DMOAC and DMOAC + FBS layers on silicone wafers was measured by variable-angle spectroscopic ellipsome-
try (VASE) using the M2000F variable-angle spectroscopic ellipsometer (J.A. Woollam Co., Inc.) The measurement was performed at 70° relative to the surface normal under ambient conditions. Ellipsometry data were fitted using a cauchy model with parameters for organic layers \( n(\lambda) = A\lambda + Bn/\lambda^2 + Cn/\lambda^4 \), with \( A_n = 1.45, B_n = 0.01, C_n = 0.0 \) to obtain dry thickness of adlayers.

### 3.3.5 Image segmentation and quantification

To limit the viability analysis to fluorescent *E. coli* and to eliminate bias in the data analysis based on GFP fluorescence intensity, fluorescence images were thresholded and segmented using the morphological strel algorithm of the image processing toolbox of MATLAB® software (MATLAB®, MathWorks; version R2010b) that combines image erosion and dilation operations. The algorithm was included into a semiautomatic image processing workflow that allows for manual adjustment of the thresholding levels of the entire time series as well as individual time frames. Binary masks were generated from the thresholded images and surface-adherent bacteria were counted automatically. The summing of binary masks from consecutive time points allowed for correction of fluorescence signal loss caused by GFP bleaching, washout and degradation of the stained DNA. To prevent false-positive results, binary masks that were not positive for the GFP channel before, were excluded from the PI positive counts to limit the analysis to bacteria that were viable initially. Elimination of x,y drift of time series data was achieved by the register virtual stack slices and transform virtual stack slices plugins of Fiji that were incorporated into a MATLAB® routine using the MIJ java package for bi-directional communication between MATLAB® and ImageJ by D. Sage. The MATLAB® file for the analysis workflow is available in the Additional file 1.

### 3.4 Results

#### 3.4.1 Impact of SYTO 9 and propidium iodide concentrations on the growth of suspended *E. coli*

To determine toxicity levels of the fluorescent DNA stains, we probed the effect of SYTO® 9 and propidium iodide (PI) on *E. coli* growth. We supplemented bacterial batch cultures in M9 growth medium with varying dye concentrations (0, 3, 30 \( \mu \)M PI, 6 \( \mu \)M SYTO® 9) and monitored bacterial growth at 37 °C by the increase in the turbidity of the solution at 600 nm (Figure 3.2 a). 6 \( \mu \)M SYTO® 9 in combination with 30 \( \mu \)M PI, as recommended in the conventional and commercial dual staining assay [26], completely inhibited *E. coli* growth. Supplementing the *E. coli* suspensions with 6 \( \mu \)M SYTO® 9
Results

Figure 3.2: Impact of SYTO® 9 and propidium iodide (PI) concentration on *E. coli* growth rate and the detection efficiency of dead bacteria in M9 bacterial medium. (a) Inhibition of bacterial growth in the presence of 6 µM SYTO® 9 and 30 µM PI. PI alone showed a dose-dependent growth inhibition. At a concentration of 30 µM *E. coli* growth was inhibited, which was not detected when the PI concentration was reduced tenfold from 30 to 3 µM. Replication of *E. coli* in 3 µM PI containing M9 medium was confirmed by growth rate measurements from a starting culture of 50% live / 50% dead *E. coli* (inset). (b) PI fluorescence of *E. coli* cultivated in M9 medium containing different PI concentrations. 3 µM PI sufficiently stained dead bacteria in a 50% live / 50% dead *E. coli* mixture, while no significant background signal increase for 3 µM PI was detected compared to the background for a 100% live bacterial solution. In contrast, supplementing the medium with 30 µM PI resulted in a significant increase of PI stained bacteria from a 100% live starting culture indicating that the high concentration of the DNA stain interferes with bacterial viability. Background fluorescence of PI supplemented M9 medium was subtracted for each of the three PI concentrations, respectively. Error bars represent the standard error of the mean.

alone showed the same growth inhibition, while 30 µM PI itself did not inhibit growth completely but did reduce the growth rate compared to the pure M9 medium. This indicates that 6 µM SYTO® 9 causes major changes in bacterial physiology. The impaired growth rate upon addition of PI was eliminated when we decreased the PI concentration in the bacterial growth medium tenfold, i.e. from 30 µM to 3 µM (Figure 3.2 a).

To confirm that the viable *E. coli* in the medium supplemented with 3 µM PI were able to replicate, we compared the turbidity increase of a 50% live / 50% isopropanol killed bacterial mixture to cultures containing 100% live and 100% isopropanol treated *E. coli* (Figure 3.2 a). Within 4 hours, the turbidity increase for the mixed 50% live / 50% dead starting culture did not reach the same level as for the 100% live culture. Those results are consistent with the expected exponential growth rate of viable bacterial batch cultures.
and thus show that the bacteria replicated normally in 3 µM PI containing medium. To determine if the reduced PI concentration was sufficient to detect dead bacteria in solution, we incubated bacterial batch cultures starting from either 100% live or 50% live / 50% killed bacteria in M9 medium containing 3 and 30 µM PI respectively. The PI fluorescence at 630 nm was subsequently measured by fluorescence spectroscopy over 3 hours (Figure 3.2 b). Supplementing the growth medium with 3 µM PI adequately stained the isopropanol treated bacteria in the 1:1 mixture of live and dead E. coli but did not result in a significant increase of the background fluorescence of the 100% live starting culture. In contrast, supplementing the medium with 30 µM PI significantly increased the PI fluorescence from the 100% live starting culture (Figure 3.2 b), which was consistent with the impaired growth rate under those conditions (Figure 3.2 a), indicating that 30 µM but not 3 µM PI is toxic to E. coli bacteria.

3.4.2 Viability and growth rate of surface-adhering E. coli is strongly reduced upon long-term incubation in culture medium supplemented with SYTO 9

For viability and growth kinetic studies of surface-adherent bacteria, E. coli that unspecifically adhered to bare glass surfaces were incubated in M9 growth medium that contained either 3 µM PI or a mixture of 6 µM SYTO® 9 and 3 µM PI (Figure 3.3 a). For SYTO® 9 containing medium, E. coli replication and surface colonization was completely blocked, as determined by time lapse video microscopy (Figure 3.3 a, Additional file 1 Figure S 3.5). In addition to inhibiting bacterial growth, the viability of surface-adhering E. coli (AAEC191A), decreased for incubation times longer than 1.5 hours, as detected by two-channel fluorescence microscopy. In contrast, no decrease of viability or impaired growth was observed for endogenously eGFP expressing E. coli (AAEC191A pHis-GFP) counterstained with 3 µM PI (Figure 3.3 a), which is in agreement to the results from the batch culture experiments (Figure 3.2). In controls we confirmed that GFP expression itself did not perturb E. coli adhesion and growth (Figure 3.4 a, b). Furthermore, the fraction of GFP fluorescent E. coli (87%) was not significantly different (α = 0.05) for eGFP expression from the IPTG inducible pHis plasmid under the control of the tac-promoter (AAEC191A pHis-GFP) and under the control of a constitutive rpsm promoter (AAEC191A rpsm-GFP) (Figure 3.4 c, d).

To evaluate whether the eGFP/3 µM PI assay is suited for in situ monitoring of bacterial viability and growth on a bioactive model substrate, eGFP-expressing E. coli (AAEC191A pHis-GFP) were incubated on antimicrobial dimethyloctadecyl [3-(trimethoxysilyl) propyl] ammonium chloride (DMOAC) coated glass surfaces [23, 27] (Figure 3.3 b). Homogeneous DMOAC coat-
3.4 Results

Figure 3.3: In situ monitoring of the growth and viability of surface-adhering *E. coli* using the eGFP/3 μM PI assay. (a) Time series of surface-adhering *E. coli* on bare glass substrates. The dual staining assay (6 μM SYTO® 9 / 30 μM PI) decreases bacterial viability on untreated glass substrates after incubation times longer than 1 hour as *E. coli* (AAEC191A) incubated with 6 μM SYTO® 9 containing medium failed to replicate (inset). In contrast, eGFP-expressing *E. coli* (AAEC191A pHis-GFP) that were incubated with 3 μM PI were able to replicate and grow on the glass surface. (b) Viability of *E. coli* (AAEC191A pHis-GFP) on antimicrobial DMOAC-coated glass surfaces as monitored by eGFP/PI fluorescence microscopy. Pre-exposure of the DMOAC surfaces to fetal bovine serum (FBS) completely blocked the antimicrobial activity. Microscopy images show the overlay of the SYTO® 9 / eGFP and PI fluorescence channels, i.e. differentiating live (green) from dead bacteria (red). 3 independent fields of view from different experiments were analyzed containing a total of 125-250 surface attached bacteria for each condition. Error bars represent the standard deviation. Scale bar 20 μm.
ing with a dry adlayer thickness of 2.2 nm was confirmed by variable-angle spectroscopic ellipsometry. To allow for a direct contact of the bacterial membrane with the surface-immobilized membrane-active DMOAC molecules, non-fimbriated eGFP expressing *E. coli* (AAEC191A pHis GFP) were used [4]. As detected by 3 µM PI staining, all adherent bacteria on the DMOAC surface were killed within 30 minutes of surface incubation and no measurable bacterial growth occurred (Figure 3.3 b, Additional file 1 Figure S 3.6, Additional file 2 Movie S1).

### 3.4.3 Pre-incubation of bioactive DMOAC surfaces with serum proteins completely blocked the antimicrobial activity and restored bacterial growth on the surface

To investigate if unspecific protein adsorption would interfere with the bactericidal activity of the DMOAC surfaces, the DMOAC surfaces were pre-incubated with fetal bovine serum (FBS) prior to bacterial incubation. Pre-conditioning of the antimicrobial surface with serum provides a model system for the rapid protein adsorption on biomaterial surfaces upon contact with host body fluids, notably blood, that can significantly impact the specific and unspecific binding of bacteria to the engineered material [10, 28]. Serum protein adsorption increased the dry adlayer thickness from 2.2 nm for pure DMOAC surfaces to 4.5 nm, as measured by ellipsometry. Using the optimized in situ eGFP/PI assay, we found that serum pre-incubation not only delayed but completely eliminated the bactericidal effect of the DMOAC surfaces on adherent *E. coli* (Figure 3.3 b, Additional file 3 Movie S2). The bacteria survived and were able to divide on the protein-coated DMOAC surfaces. Division times of the surface-attached *E. coli* were comparable to those on bare control glass surfaces without bactericidal activity (Figure 3.3 a).

### 3.5 Discussion

A fluorescence based assay is introduced here that is well suited for the *in situ* monitoring of the viability and growth kinetics of surface-adhering and suspended bacteria. While we used *E. coli* as model organism, this assay should be applicable to other bacterial species as well if (i) the commonly used live DNA stains, such as SYTO® 9, are replaced by endogenous eGFP expression and (ii) if the concentrations of propidium iodide (PI) needed to detect dead bacteria is reduced to non-toxic levels (3 µM for *E. coli*). While most available viability assays are restricted to suspended bacteria (i.e. CFU assay) or optical endpoint determinations (i.e. SYTO® 9/PI LIVE/DEAD BacLight™ viability kit, CTC assays), we show by fluorescence time-lapse
3.5 Discussion

Figure 3.4: Influence of GFP expression on the adhesion and fluorescence of *E. coli* K-12 derivative strains. (a) The number of *E. coli* adhering to bare glass substrates was analyzed for the non-fimbriated empty strain *E. coli* AAEC191A, strain AAEC191A pHis-GFP that expresses GFP from pHis plasmid upon IPTG induction and strain AAEC191A rpsm-GFP carrying a plasmid to express GFP from the constitutive rpsm promoter. Per strain, bacteria from 20 fields of view (each 200×200 μm) were analyzed. Mean and standard deviation are shown and a two-independent sample two-sided t-test (α = 0.05) was performed. For the *E. coli* strains tested, the number of adherent bacteria was not significantly different (n.s.) with and without GFP expression. Population variances were not significantly different as tested by a two-sided F-test (α = 0.05). (b) Growth curves of *E. coli* K-12 derivative strains with and without plasmids for GFP expression. Turbidity of bacterial suspensions in 96 well plates was measured at 600 nm. Mean and standard deviation of a triplicate measurement are shown. (c,d) The fraction of adherent, GFP-fluorescent *E. coli* was analyzed. The empty strain (AAEC191A, n = 2468) was not fluorescent. GFP expression from both inducible and constitutive promoters yielded similar fractions of fluorescent *E. coli* (87.3% and 87.2%, respectively). A total of 2852 bacteria carrying the inducible *gfp* gene (pHis-GFP) and a total of 2036 bacteria carrying the constitutive *gfp* gene (rpsm-GFP) were analyzed. Shown are means and standard deviations. A two-independent sample two-sided t-test (α = 0.05) was performed and the fraction of fluorescent bacteria was not significantly different (n.s.) for the different GFP expressing *E. coli* strains. Population variances were not significantly different as tested by a two-sided F-test (α = 0.05).
microscopy and turbidity measurements, that reducing the PI concentration to 3 µM readily stained dead *E. coli* on bioactive DMOAC surfaces without disturbing bacterial growth (Figure 3.2, Figure 3.3).

Our assay allows viability monitoring of single bacteria and emerging bacterial colonies. We should note though, that the assay is not directly transferable to the study of mature biofilms without additional calibrations since the metabolic and genetic profile changes during biofilm mode of growth [29–31] and the synthesis of extracellular polymeric substances (EPS) [32, 33] might influence the bacterial GFP expression as well as the passive diffusion of PI through the biofilm matrix. Furthermore, detection of single bacteria within a dense three-dimensional biofilm matrix by epifluorescence microscopy might be challenging. However, since bacterial surface colonization starts with the adhesion of individual bacteria, the presented assay provides a versatile new tool for high spatial and temporal evaluations of bacterial viability on engineered surface coatings. The assay thus adds to the previously reported eGFP/PI flow cytometry assay, that was limited to viability determination of suspended bacteria [21] and to the eGFP/PI endpoint viability study of groundwater *E. coli* [34].

Evaluating bacterial viability on the test surface omits the extraction of the adherent bacteria as required for solution based assays e.g. CFU counts. Therefore, testing the bacterial viability on the substrate might increase the reliability of the assay since extraction is commonly achieved by ultrasonication or harsh washing procedures, both of which can harm bacteria. Furthermore, all CFU assays require prolonged incubation times for colony growth and are thus not applicable for real-time viability monitoring. As an alternative to extraction, an agar sandwich assay has been suggested to determine the viability of surface-adherent bacteria [35]. This method, however, is prone to errors, since each transferred bacterium will grow into a colony that in turn might overlap with colonies nearby. As an alternative direct optical viability assay of surface-adherent bacteria, the respiratory potential of bacteria can be monitored using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) [17]. The drawback however is that the CTC stain cannot be used for real-time monitoring since CTC disrupts the respiratory chain and is toxic to bacteria. This makes the CTC assay only suitable for end point determinations. We compared the performance of our eGFP/PI assay to the well-established SYTO® 9/PI endpoint dual staining assay and found identical detection efficiencies of dead *E. coli* (Additional file 1 Figure S 3.6). The SYTO® 9/PI assay itself has been extensively compared to the above-mentioned viability tests [17, 19, 21, 26, 36] and showed comparative results to the solution based CFU assay as well as to other microscopy based endpoint viability protocols including the CTC assay. The added advantage of our assay is the ability to monitor the viability of adherent bacteria in real-time.
For each bacterial strain and species, one needs to optimize the PI concentrations to keep the bacteria viable, as done here for *Escherichia coli* (*E. coli*) K-12 MG1655. *E. coli* K-12 derivatives have been widely used as model strains in surface adhesion and biofilm studies [4, 31, 37–39]. We expressed eGFP from the pHis plasmid under the control of the IPTG inducible tac-promoter to replace the growth inhibiting DNA stain SYTO® 9 as live bacterial marker. To exclude negative effects of GFP expression as well as IPTG and antibiotic addition on bacterial viability and adhesion, we compared the adhesion properties and growth kinetics of the K-12 AAEC191A background strain [24] to a constitutive eGFP expressing strain (rpsm-GFP) and the IPTG inducible pHis-GFP strain (Figure 3.4 a, b). No significant difference in adhesion and growth was observed. Furthermore, no significant difference in the fraction of GFP-expressing bacteria was found for the eGFP expression under the constitutive and inducible promoter used in this study (Figure 3.4 c, d). However, even non-toxic gene products like GFP can have detrimental effects on bacteria when overexpressed [40], since overexpression of an introduced gene requires a lot of resources and thus might disorganize the bacterial metabolism. Therefore, the inducible IPTG-based expression system, as compared to constitutive expression systems, allows for a control of GFP expression and guaranteed a balance between fluorescent and healthy bacteria (Figure 3.4 c, d). Since eGFP is a very stable protein [41, 42] new GFP variants with reduced half-lives, e.g. GFP(LVA), have been suggested to study transient gene expression in bacteria [43]. The enzymatic degradation of unstable GFP(LVA) however, requires a metabolically active viable bacterium (Additional file 1 Figure S3.7). Thus, the use of GFP(LVA) variants does not improve our assay to limit false-positive detection of dead bacteria.

For live cell imaging, GFP expression in bacteria is used extensively and expression systems are available for many different bacterial species, including clinically relevant strains of *Salmonella*, *Streptococcus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* O157:H7 [41, 44–47]. Plasmid based gene expression is a well-established and long-used method in microbiology. Handling of plasmids is usually easy and versatile and genes, promoters or selection markers can quickly be exchanged and adapted to needs. Compared to plasmids, chromosomal insertions are more complicated and cannot be adapted as easily. Depending on the insertion method used, the gene of interest is inserted in the chromosome at a random location and might disrupt an important chromosomal gene. As for plasmids, selection markers like antibiotic resistances are commonly used for chromosomal insertions, too [48]. Furthermore, chromosomal insertions usually result in the insertion of a single copy of the gene into the chromosome. The pHis plasmid used in this study carries a ColE1-like replicator and occurs at nearly 20 copies per bacterium [49], each providing the gene of interest. This
results in very stable and usually higher expression levels of the gene of interest (here eGFP) than with chromosomal insertions. Plasmids with ColE1-like promoters are stably inherited even without the presence of the corresponding selection agent [50, 51], long-term experiments with GFP expression from a plasmid rather than from a chromosomal insertion are feasible for more than a few hours. For other bacterial species and expression systems, the IPTG level and the concentration of the antibiotic selection marker should be re-evaluated to assure a stable GFP expression without disturbance of bacterial growth.

Finally, we applied our viability assay to highlight the impact of protein adsorption on the antimicrobial activity of engineered DMOAC surfaces. Upon incubation of the DMOAC surfaces with protein-rich fetal bovine serum, bacterial growth on the otherwise bactericidal surface was possible, indicating that the protein layer on top blocked the bioactive quaternary ammonium groups of the DMOAC coating (Figure 3.3 b). The growth rate of the surface-attached \textit{E. coli} on the control glass and serum-coated DMOAC substrates were identical, illustrating that the design rules for antimicrobial coatings primarily have to be tuned to prevent both, bacterial and protein adsorption since additional bioactive modifications can be lost when the biomaterial gets in contact with protein-rich (host) fluids.

3.6 Conclusions

In conclusion, we show that the eGFP/PI assay is suited to study the antimicrobial properties of (bio-) material surface coatings under physiological conditions in real time and with single-bacterium sensitivity. This was so far not possible with the widely used solution based assays (i.e. CFU) or endpoint dual staining protocols (i.e. LIVE/DEAD BacLight\textsuperscript{TM} viability kit, CTC). Possible applications for the assay include studies of bacterial fitness and pathogenicity on biomaterial surfaces using live cell imaging of bacteria as additional readout. While we calibrated and illustrated the advantages of the assay for \textit{E. coli}, other PI concentrations might have to be employed to optimize the kinetic viability monitoring of other bacterial species. While conventional bacteria viability assays allow for fast endpoint checks without requiring genetic modifications, the eGFP/PI assay presented here constitutes a viability test procedure that requires only one sample and its replicates per time series and is particularly suited for kinetic studies.

Competing interests

The authors declare that they have no competing interests.
Acknowledgments

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3 Kinetic viability assay

Supplementary figures

**Figure S 3.5:** Protein coating reconstitutes bacterial growth on bioactive DMOAC surfaces as measured by an increase in bacterial surface coverage. The growth kinetics of 5-15 surface attached bacteria were analyzed and averaged for each condition. Error bars represent the standard deviation.

**Figure S 3.6:** The eGFP/PI and the SYTO® 9/PI dual staining assays yield identical detection efficiencies of *E. coli* viability on bioactive DMOAC surfaces with fast bacteria deactivation kinetics (complete bacterial killing within 1 h incubation).
3.6 Conclusions

Figure S 3.7: Enzymatic degradation of GFP variants with different stability. All *E. coli* strains express GFP from plasmid pHis under control of the inducible tac-promoter. At time point 0 h GFP expression was stopped by removing the IPTG inducer. *E. coli* expressing the stable eGFP variant showed the highest fluorescence intensity and nearly no degradation within 4.5 h. Strains that expressed the unstable GFP(LVA) variant exhibited an inherent lower fluorescence intensity from the start, as the unstable GFP(LVA) was constantly being degraded by innate *E. coli* proteases. When GFP(LVA) expression was stopped by IPTG removal and the culture is maintained at 37 °C, the GFP fluorescence decreased rapidly, indicating that the GFP(LVA) is degraded enzymatically. If the culture was kept at 0 °C after IPTG removal, no degradation of the GFP(LVA) was observed. All measurements were performed in M9 minimal medium. 1 ml samples were drawn at each time point and measured with a Perkin Elmer spectrophotometer. OD600 of all cultures at 0 h was set to 1.
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References


4 Bacterial filamentation accelerates colonization of adhesive spots embedded in biopassive surfaces

This chapter 4 was published in New Journal of Physics 15 (2013) 125016 under the terms of the Creative Commons Attribution 3.0 license\(^1\). J. Möller, P. Emge and V. Vogel designed the experiments. P. Emge and I. Avalos Vizcarra performed the experiments, P. Kollmannsberger developed and performed the mathematical model. P. Emge and P. Kollmannsberger analyzed the results. All authors contributed to the writing and the discussion of the manuscript. P. Emge and J. Möller contributed equally to this work.

4.1 Abstract

Sessile bacteria adhere to engineered surfaces and host tissues and pose a substantial clinical and economical risk when growing into biofilms. Most engineered and biological interfaces are of chemically heterogeneous nature and provide adhesive islands for bacterial attachment and growth. To mimic either defects in a surface coating of biomedical implants or heterogeneities within mucosal layers (Peyer’s patches), we embedded micrometer-sized adhesive islands in a poly(ethylene glycol) (PEG) biopassive background. We show experimentally and computationally that filamentation of *Escherichia coli* can significantly accelerate the bacterial surface colonization under physiological flow conditions. Filamentation can thus provide an advantage to a bacterial population to bridge non-adhesive distances exceeding 5 \(\mu\)m. Bacterial filamentation, caused by blocking of bacterial division, is common among bacterial species and can be triggered by environmental conditions or antibiotic treatment. While great awareness exists that the build-up of antibiotic resistance serves as intrinsic survival strategy, we show here that antibiotic treatment can actually promote surface colonization by triggering filamentation, which in turn prevents daughter cells from being washed away. Our combined microfabrication and computational approaches provide quantitative insights into mechanisms that enable biofouling of biopassive surfaces.

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with embedded adhesive spots, even for spot distances that are multiples of the bacterial length.

4.2 Introduction

Bacterial attachment is the first step in the colonization of surfaces and biofilm growth. As the preferred mode of microbial life, biofilms consist of surface attached and densely packed populations of bacteria, held together by a self-produced matrix [1]. Biofilms growing on medical devices and biomedical implants are generally accepted to be the major cause of bacterial infections in clinical environments [2], causing tremendous clinical and economical complications [3]. Commonly, bacterial infections are treated by antibiotic therapies, but due to an increasing number of antibiotic-resistant bacterial strains, pharmaceutical agents become ineffective and new ways to prevent bacteria borne infections need to be explored [4–6]. One alternative strategy to deal with bacteria-related infections is to interfere with the initial adhesion of planktonic (free floating) bacteria to biomedical and technical surfaces by rendering the surfaces biopassive [7–11]. Upon exposure to the environment however, biopassive coatings can be subjected to degradation and corrosion [12]. Defects within the antimicrobial coating in turn can provide adhesion sites for bacteria serving as nucleation points for the formation of confluent surface biofilms. Bacteria not only adhere to and colonize engineered materials. To invade the host, pathogens adhere to host tissues by specific surface receptor-ligand interactions. The mammalian intestine is a preferred area of infection as it provides a large epithelial surface that can be colonized by potentially pathogenic microorganisms [13]. Within the intestine epithelium, Peyer’s patches, i.e. groups of lymphoid follicles, provide a transport system for antigens as an essential part in the immune defence. Several pathogenic bacteria bind preferentially to cell types found in Peyer’s patches [14] as the non-adhesive mucous layer is thin compared to the surrounding tissue, making Peyer’s patches a preferred site for invasion of pathogens into the host tissue [15]. Peyer’s patches within the intestine epithelium therefore have similar properties like the adhesive defects in antimicrobial coatings described above: they present preferred sites for bacterial attachment within a nonadhesive surface.

To form a confluent bacterial layer as a requisite for biofilm development, the adherent bacteria have to bridge the non-adhesive areas on those heterogeneously adhesive interfaces. *Escherichia coli* (*E. coli*), one of the best-studied intestinal microorganisms, are rod-shaped bacteria with a length of 2-4 µm [16]. However, the morphology and in particular the aspect ratio of *E. coli* can largely change upon exposure to antibiotics and non-optimal growth con-
Filamentation occurs when cell division is blocked while growth continues, resulting in filaments with high aspect ratios of several tens of micrometres. As a survival strategy, filamentation can slow down the phagocytic uptake of bacteria by immune cells [18], promoting their survival within host tissues [19, 20]. Since bacterial filaments are commonly observed in patients being treated with antibiotics [21], it has been proposed that filamentation might be a defence mechanism to temporarily withstand treatment with β-lactam antibiotics, e.g. cephalaxin [22, 23]. Cephalaxin belongs to the family of β-lactams and targets FtsI, a penicillin binding protein of the divisome complex that is required for bacterial division. It is among the largest selling antibiotics worldwide and is applied against otitis media and urinary tract infections.

Since the exposure to antibiotics or the local growth conditions that promote filamentation can change quickly in natural environments, we ask here whether filamentation offers a selective advantage to colonize heterogeneously adhesive surfaces. Using *E. coli* as model organism, we hypothesize that filamentous bacteria bridge non-adhesive regions between adhesive spots faster than non-filamentous bacteria. To test our hypothesis we performed live cell microscopy and analysed the kinetics of *E. coli* adhesion and growth on heterogeneous surfaces. To allow for firm adhesion of *E. coli* under physiological flow conditions [24, 25], we used a photoresist lift-off process to micropattern the glycoprotein Ribonuclease B (RNaseB) to which *E. coli* bind by their type 1 fimbriae. Adhesive RNaseB islands of 10 µm in diameter were spaced at distances reaching many multiples of the bacterial length. Unspecific adhesion was blocked by passivation with PLL-g-PEG [9, 26]. *E. coli* filamentation was induced by cephalaxin. Based on the experimentally derived parameters of bacterial adhesion and growth, we implemented a Monte Carlo simulation of bacterial surface colonization to confirm the experimental data and obtained additional predictions for the impact of filamentation, flow and adhesive spot distances on the bacterial surface colonization kinetics. This study provides novel mechanistic insights into the initial stages of surface biofouling and it highlights the selective advantage of bacterial shape adaptation to colonize heterogeneously adhesive substrates.

### 4.3 Materials and Methods

#### 4.3.1 Bacteria

*E. coli* AAEC191A (∆fim null mutant) [27] harbouring plasmid pSH2 [28] expressing a naturally occurring uropathogenic FimH variant were grown overnight at 37 °C in an orbital shaking incubator in lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) with 10 µg/mL
chloramphenicol to maintain plasmid pSH2. For flow chamber experiments, the culture was washed twice in phosphate-buffered saline (PBS, pH 7.4) and adjusted to OD$_{600}$=1.

4.3.2 Preparation of adhesive glycoprotein patterns and unpatterned adhesive surfaces

RNaseB glycoprotein patterns [29] with a diameter of 10 µm, spaced by 5, 10, and 20 µm were prepared using a combined photolithography and lift off process (Figure 4.1). The MAPL protocol [30] was adapted to pattern proteins onto glass substrates. Photoresist patterns on glass cover slides were created using positive S1818 photoresist. Briefly, undiluted S1818 photoresist (Microposit) was spin coated on the cover glasses in a two-step spin process with 2000 rpm for 5 seconds (ramp step of 500 rpm/s) and 4000 rpm for 90 seconds (ramp step of 800 rpm/s), respectively, followed by soft baking for 60 seconds at 100 °C and UV light exposure (Karl Süss MA6 mask aligner) with an energy dose of 150 mW/cm$^2$ at 405 nm. Exposed resist was developed in undiluted MF319 developer (Microposit) for 60 seconds. The resist micropatterns were rendered biopassive by 1 hour incubation at room temperature with 100 µg/mL PLL-g[3.5]-PEG(2) (SuSoS AG) in PBS. For enhanced stability of the polymer layer, the glass surface was coated with 21 nm niobium oxide prior to photolithography. The photoresist was removed by chemical lift off with N-Methyl-2-pyrrolidone (NMP, Sigma 494496), leaving PLL-g-PEG coated regions on the surface. The non-PEGylated areas were backfilled by 30 minutes incubation at room temperature with 100 µg/mL Alexa Fluor 488 labelled RNaseB. The terminal mannoses of the N-linked glycans present on RNaseB serve as receptor for the bacterial type 1 fimbriae tip adhesin FimH. The biopassive properties of PLL-g-PEG prevent the adsorption of RNaseB to previously passivated areas [26]. The unpatterned adhesive surfaces were prepared by incubating glass slides 30 minutes at room temperature with 100 µg/mL unlabelled RNaseB.

4.3.3 Parallel plate flow chamber experiments

The flow chamber comprised of a vacuum mediated assembly of an acrylic top, a silicone separating gasket and a glass cover slide. A channel of 2.5 mm width was cut into the silicone gasket. The channel height was defined by the gasket thickness of 0.254 mm. The channel dimensions and the volumetric flow rate of 1 mL/min defined a shear stress of 0.6 pN/µm$^2$ applied to the surface-bound E. coli. The flow cell setup was placed within a microscope incubation box heated to 37 °C. A custom-made bubble trap that was incorporated upstream of the flow chamber inlet reduced the probability of air bubbles running into
the flow chamber. LB medium supplemented with 10 µg/mL chloramphenicol was circulated through the flow chamber setup for at least 1 hour prior to each experiment to equilibrate the system. To seed the glass surface with bacteria, the flow was stopped, the tubing clamped and disconnected at the flow chamber inlet port. A syringe with the inoculation culture was used to inject the bacteria directly into the inlet port of the flow chamber. The bacteria were allowed to adhere to the surface for 1 minute. The tubing was reconnected and the flow resumed. Unbound bacteria were washed off by the medium flow. Bacterial filamentation was induced by supplementing the LB medium with 20 µg/mL cephalixin. To restore bacterial division, the medium was exchanged with cephalixin-free LB medium. The medium change led to gradual dilution of the cephalixin in the setup over time.

4.3.4 Microscopy

Flow chamber assays were performed on an inverted live cell microscope (Nikon TE2000-E) equipped with an electron multiplying charge-coupled device (EM-CCD) camera (Hamamatsu 9100-02). Time-lapse movies of multiple stage positions were acquired every 3 minutes for up to 4 hours with a 40x phase contrast objective. Fluorescence images were acquired with a fluorescein isothiocyanate (FITC) bandpass filter set (Chroma 49002).

4.3.5 Data analysis

Surface colonization kinetics

To determine the colonized area fraction, phase contrast images were thresholded within a rectangular region of interest including multiple adhesive spots in the direction of flow. To include bacterial growth perpendicular to the flow, the region of interest was 2 times wider than the spot width and centred over the spots (Figure 4.2 b). The area fraction is calculated as the ratio of thresholded pixels by the total number of pixels in the region of interest. To assess differences in the initial surface coverage, individual plots were fitted and the parameters obtained for generation times were corrected.

Bacterial bridging of passivated areas between adhesive spots

The position of the adhesive spots was determined from fluorescent images of Alexa Fluor 488 labelled RNaseB. A position mask of the adhesive islands was created and overlaid with the phase contrast images to visualize the adhesive spots in the images and time-lapse movies. Bridge advancement was measured from phase contrast images in between two neighbouring spots in flow direction. Bacteria growing beyond the border of an upstream spot towards
4 Bacterial surface colonization

an adjacent downstream spot contributed to the bridge advancement. Pairs of adhesive spots (n=10) were randomly chosen and the bridge advancement was determined by measuring the distance that the bacteria advanced from the border of the upstream spot towards the downstream spot. The maximum distance of bridge advancement was the edge-to-edge distance between two spots (5, 10 or 20 µm, respectively). Bridging time was defined as the time point when bacteria from an upstream adhesive spot connected to the next downstream spot. Analysis of E. coli filaments was restricted to the time interval where filamentation occurs and before bacteria lysed.

Bacterial length doubling time measurements

For filamentous E. coli, the time a filament needed to double its length was considered to be an adequate measure of growth and was determined from the pole-to-pole length of a filament. The filament length (n=10) was measured every 3 minutes for 2 hours. The data was plotted and fitted with theoretical growth curves to obtain a length doubling time. To compare the length doubling time of filaments with non-filamentous E. coli, the pole-to-pole length of all progeny of non-filamentous E. coli (n=10) was summed up at each time point and a length doubling time was determined likewise. Data analysis was performed using ImageJ software.

Computational modelling of surface colonization kinetics

2D Monte Carlo simulations of a model describing the bacterial growth on adhesive spots were performed using MATLAB (MathWorks, version R2010b). The surface was modelled as a lattice in which bacteria occupy one lattice site, corresponding to an area of 1 µm². The parameters of the model were the doubling time T_d of filamentous and non-filamentous E. coli as derived from experimental data, a probability P_{filament} for growth without septation, resulting in filaments, a probability P_{divide} to account for septation (as observed in undisturbed bacterial growth as well as for filaments when removing cephalexin antibiotics), and a probability P_{wash-off} for bacteria being washed off from the edge of the colony over biopassivated surface areas. The wash-off probability depends on the magnitude of flow, modelled by a flow factor F, and the position of the bacteria relative to the flow direction, described by an anisotropy factor A. A more detailed description of the model and implementation is found in the supplementary information.
4.4 Results

4.4.1 Increasing distances of adhesive spots inhibit the formation of a confluent layer of non-filamentous *E. coli*

To quantify the surface colonization kinetics of non-filamentous *E. coli* on heterogeneously adhesive surfaces, we prepared mannosylated RNaseB glycoprotein patterns within a stable biopassive PLL-g-PEG layer [9, 31] by adaptation of a combined photolithography and molecular assembly process (Figure 4.1) [30]. The lift off process rendered the glass surfaces with a distinct pattern of circular RNaseB spots as it was visualized by fluorescence microscopy. No fluorescent signal was detected within the biopassive PLL-g-PEG coated areas (Figure 4.1, iv). The RNaseB pattern promoted firm adhesion of type 1 fimbriated *E. coli* under physiological flow conditions [24] and no bacterial adhesion to the passivated areas was observed (Figure 4.1, v).

To analyse the effect of surface patterning on the kinetics of bacterial surface colonization, *E. coli* were incubated on adhesive RNaseB spots of 10 µm in diameter separated by distances of 5, 10 and 20 µm. Surface-attached *E. coli* were grown under physiological relevant shear stress (1 ml/min flow rate; 0.6 pN/µm² wall shear stress) [32–34]. The surface colonization kinetics on the different patterns was quantified from time-lapse movies for a region of interest that included multiple adhesive spots within the direction of medium flow (Figure 4.2 b, SI movie S1 and S2). Starting from single adherent *E. coli*, bacterial microcolonies grew in size by cell division until the edge of an adhesive spot was reached. An almost confluent bacterial layer was observed after 4 hours for a spot distance of 5 µm (Figure 4.2 a, top). With increased spot distances of 10 and 20 20 µm, the bacterial surface coverage decreased and characteristic colonization patterns were observed (Figure 4.2 a, middle and bottom). *E. coli* microcolonies on unpatterned adhesive surfaces merged into a confluent bacterial layer after 4 hours (Figure 4.2 b). Non-filamentous *E. coli* colonized surfaces with 5 and 10 µm spot distances are very similar to unpatterned adhesive RNaseB surfaces. No confluent layer had formed on surfaces with 20 µm spot distances (Figure 4.2 c). Since the initial number of adherent *E. coli* per field of view was higher for surfaces with higher adhesive spot densities, we corrected the colonization kinetics analysis accordingly. By image thresholding of the phase contrast time-lapse movies, the fractions of pixels covered by bacteria were determined. This analysis was very robust for low to moderate bacterial surface coverage. At higher surface coverage though, light halos around the bacteria occurred such that the phase contrast imaging and thresholding was not an accurate measurement of surface coverage any longer. We estimated that a pixel threshold of 58% corresponds to a confluent *E. coli* monolayer (Figure 4.2 c).
Figure 4.1: Patterning surfaces with bacteria-adhesive islands within a biopassive surface coating. (i) S1818 photoresist structures (red) were fabricated by photolithography on Nb$_2$O$_5$ coated glass cover slides. (ii) Incubation of photoresist-patterned substrate with PLL-g[3.5]-PEG(2). (iii) Photoresist lift-off by N-Methyl-2-pyrrolidone. (iv) Backfill of non-PEGylated regions with the mannose-exposing glycoprotein RNaseB, labelled with Alexa Fluor 488 for visualization. (v) E. coli expose the adhesin FimH at the tip of type 1 fimbriae and adhere to the RNaseB pattern by specific FimH-mannose interactions but not to the biopassive PLL-g-PEG coated regions. The corresponding images of the photoresist pattern before the lift-off process and Alexa Fluor 488 labelled RNaseB patterns are shown (circular 10 µm spots with 20 µm spot-to-spot distance). Bacteria adhere selectively to the adhesive spots under fluid flow conditions as shown here for a flow rate of 1 mL/min.
4.4 Results

Figure 4.2: *E. coli* colonization kinetics on micropatterned surfaces under physiological flow. (a) Phase contrast images of *E. coli* on RNaseB spots with different distances after 4 h growth at 37 °C with a flow rate of 1 mL/min (0.6 pN/µm² shear stress). Distances of the adhesive spots and flow direction are indicated. Dashed circles visualize the location of the adhesive spots. (b) Growth kinetics of *E. coli* on patterned and unpatterned surfaces. The bacteria-covered area was determined by thresholding phase contrast images. The region of interest was defined by a row of adhesive spots in the flow direction (red false coloured). Exemplary image sequences show the bacterial growth kinetics on a 10 µm circular pattern with 20 µm spot-to-spot distance (top panel) and on an unpatterned surface (bottom panel). Only a part of the actual analysed area is shown. (c) Area fractions of bacterial surface coverage. Standard errors of the mean are shown (n=5). A confluent *E. coli* monolayer on the unpatterned surface was estimated to correspond to an area fraction of 0.58 (indicated in the graph by a grey bar) based on the thresholding method of the phase contrast images.
4 Bacterial surface colonization

4.4.2 *E. coli* filamentation accelerates bridging of non-adhesive areas

While bacterial filamentation can be induced by various environmental factors including low temperature and high pressure [21, 35, 36], we induced *E. coli* filamentation here by inhibiting cell septation by the antibiotic cephalexin (20 µg/mL) (Figure 4.3 a, top panel and SI movie S3) [23, 37]. The filaments started to lyse 2 hours after continued exposure to cephalexin, which defined the time window of our investigation. The time for a bacterial filament to double in length was 25.4±1.6 minutes (n=10). The filament growth rate was not significantly different from the length doubling time of non-filamentous *E. coli* (27.6±3.4 min, n=10, Figure S 4.8).

To study the impact of bacterial filamentation on the bridging of non-adhesive regions, we compared the bridging of non-adhesive spot-to-spot distances of 5, 10 or 20 µm for filamentous and non-filamentous *E. coli*. Non-filamentous *E. coli* divided and grew on the adhesive patterns until the edge of the spots was reached (Figure 4.3 a). Once the spot edge was reached, we observed that daughter cells that grew into the passivated area were frequently washed away by the fluid flow after the division was completed (Figure 4.3 a, bottom panel and SI movie S4). This behaviour led to a characteristic “shark-tooth” pattern in the bridge advancement (Figure 4.3 a, b, blue lines and arrow 1 and 2). When an adhesive spot became crowded after 2-2.5 hours, daughter cells were no longer washed away and the bridge advancement accelerated until the full distance of 20 µm to the downstream patch was bridged (Figure 4.3 a). Analysing the bridge advancement for different pairs of adhesive spots revealed substantial heterogeneity in the colonization kinetics of non-filamentous *E. coli* (Figure 4.3 b, blue lines showing 3 representative measurements). In contrast, filaments were able to grow and extend across the nonadhesive areas beyond the pattern edges (Figure 4.3 b). As the filaments grew, no “shark-tooth” patterns in the advancement kinetics of the filamentous bacterial bridge were seen since no daughter cells were washed away (Figure 4.3 b, red lines and Figure 4.3 a, top panel). For 20 µm spot-to-spot distances, filamentous *E. coli* bridged the non-adhesive regions 3 times faster than non-filamentous bacteria (Figure 4.3 b).

For spot distances of 5 and 10 µm, fast bridging between the neighbouring adhesive spots was observed for filamentous and non-filamentous *E. coli* (Figure 4.3 c, d). For filamentous *E. coli*, the time to bridge the non-adhesive regions changed proportionally to the spot distance as no daughter cells are washed off (Figure 4.3 e). For non-filamentous *E. coli*, the bridging time did not scale with the spot distance. Non-filamentous *E. coli* bridged the 5 µm distance non-proportionally faster than the 10 and 20 µm (Figure 4.3 e). The bridging time, i.e. the time that filamentous and non-filamentous *E. coli*
needed to fully bridge the distance between two adhesive spots, was statistically significantly different at all spot distances, however, at 10 and 20 µm spot distance the difference was highly significant (p<0.001) (Figure 4.3 e).

4.4.3 Filament bridges disintegrate when removing cephalexin from the medium

To mimic the effect of fluctuating growth conditions, we asked how the removal of antibiotics from the cell medium might affect the further colonization. In the presence of cephalexin, E. coli filaments extended beyond the pattern edge and once the non-adhesive region was bridged, the filament tip adhered to the adjacent downstream adhesive spot (Figure 4.4 a and SI movie S5). Being fixed at both ends, further growth resulted in buckling of the filament as indicated by the filament being out of focus. Bacterial division resumed when cephalexin was reduced to sub-inhibitory concentrations (lower than 10 µg/mL[37]), which leads to a rapid fragmentation of the filaments. After cephalexin withdrawal, the time-lapse series shows how the bacterium at the filament tip that contacted the downstream adhesive spot (Figure 4.4 a, 2:12 h, arrow) remained attached on the surface and started to divide (Figure 4.4 a, 2:12-2:54 h). The non-adhesive parts of the fragmented filament are washed away (Figure 4.4 a, 2:54 h). The growth of the filaments was not restricted by the medium flow direction (Figure 4.4). We observed filaments that grew both in and against the flow direction (Figure 4.4 a, 1:42 h-1:51 h and SI movie S5). A second filament grew against the flow direction towards the downstream patch but detached after 2 h(SI movie S5) and was washed away by the fluid flow. On a homogenously adhesive area, the filaments divided into progeny over the entire length of the filament after the exchange to cephalexin-free medium and grew into a microcolony on the surface (Figure 4.4 b and SI movie S6).

4.4.4 A computational model of bacterial surface colonization predicts a kinetic advantage of filaments when colonizing passivated surfaces that contain adhesive spots

The results obtained by time-lapse microscopy showed that bacterial filamentation accelerated the bridging of neighbouring adhesive spots (Figure 4.3). To evaluate if the kinetic advantage of bridging at early stages results in increased surface coverage at later times, a 2D computational model of bacterial growth was developed that accounted for filamentous and non-filamentous bacterial growth on micropatterned adhesive surfaces.

Four basic model assumptions were made that were derived from our experimental data: (i) All bacteria grow with a constant doubling time $T_d$ of
Bacterial filamentation accelerates bridging of non-adhesive regions. (a) Time series of surface colonization for a single spot pair at a distance of 20 µm for filamentous and non-filamentous *E. coli*. The bridged distance was measured from the border of the downstream patch to the outermost bacterium contributing to the bridge advancement (dashed red and blue lines) and analysed over time. Prolonged exposure to cephalexin resulted in the lysis of bacteria (data not shown). Flow direction, volumetric flow rate and applied shear stress are indicated. (b) The plot shows measurements of three representative patch pairs for each filamentous (red) and non-filamentous (blue) *E. coli*. The dashed red and blue lines correspond to the data from the image series shown in (a). The black dashed line in the plot indicates the full distance between the spots. Detachment of fully segmented daughter cells of non-filamentous *E. coli* led to a shark-tooth pattern (arrow 1 and 2). (c) Three representative measurements for filamentous (red) and non-filamentous (blue) *E. coli* for spot distances of 5 µm (c) and 10 µm (d). (e) Bridging time of different spot distances. Mean values (n=10) and standard deviations are shown. A two-sided two-sample t-test was performed showing that the means were significantly different. **: p<0.01; ***: p<0.001.
4.4 Results

Figure 4.4: *E. coli* filaments grow in the presence of antibiotics and disintegrate after cephalexin removal, leaving behind their offspring on the adhesive RNaseB spots (a) Time series of cephalexin-induced filament growth on patterned surface under fluid flow (1 mL/min; 0.6 pN/µm²). Filamentous *E. coli* bridged passivated areas between adhesive spots (dashed circles) reaching downstream spots to which they can adhere. Continued growth of the filament between adhesive spots resulted in buckling of the filament bending it out of the focal plane. After gradually purging with cephalexin-free medium, cell division was resumed and the filaments fragmented. Only bacteria at the filament tip that were already in contact with the adhesive spots were able to hold on and further divide. A second filament is seen to grow against the flow direction (1:42-1:52 h) before it detached and was removed by fluid flow after 2 h. (b) Time series of filament growth on adhesive unstructured surfaces. Filaments can also be seen to grow at angles to the flow direction. Once cell division was restored, the filament fragmented into single bacteria over its entire length. The time point where the cephalexin was diluted below inhibitory concentration is indicated. Arrows indicate cell division events. The complete time series for (a) and (b) are shown in SI movies S5 and S6, respectively.
4 Bacterial surface colonization

26 minutes. This parameter was derived from our finding that the elongation rates did not differ significantly between filamentous and non-filamentous bacteria (Figure S4.8). (ii) Filamentation of bacteria occurs with a probability $P_{\text{filament}}$, while existing filaments can divide with a probability $P_{\text{divide}}$. These two parameters were based on our experimental observation that cephalexin induced a homogenously filamenting population which could be switched back to non-filamentous growth when cephalexin was diluted out (Figure 4.4). (iii) Bacteria at the edge of the colony are washed off with a probability $P_{\text{wash-off}}$ that depends on the magnitude of flow and the orientation of the bacteria relative to the flow direction. The two parameters magnitude and orientation are represented by a flow factor $F$ and an anisotropy factor (see supplementary information). (iv) Wash-off can only occur over non-adhesive regions. This model assumption is valid since bacteria firmly adhered to the adhesive RNaseB spots (Figure 4.3 b).

We applied our model to adhesive spot distances of 5, 10 and 20 µm as we probed experimentally. Three representative curves of filamenting (red) and non-filamenting (blue) conditions for a spot distance of 20 µm are shown (Figure 4.5 a). Our model predicts a 3 times faster bridging for filamentous compared to non-filamentous conditions, which is in good agreement with our experimental observations (Figure 4.3 a). Since the model reproduces the experimental results for 5, 10 and 20 µm distances, we applied it to larger distances of 40 and 80 µm (Figure 4.5 b). The model predicts an increasing difference in bridging time between filamentous and non-filamentous bacteria for larger spot distances (Figure 4.5 b). To gain insight into the effect of the flow factor $F$ on the bridging time, we varied the flow factor from 30 to 300 (Figure 4.5 c). Higher flow factors resulted in an increased bridging time of a 20 µm distance for non-filamentous bacteria (Figure 4.5 c, blue bars). Interestingly, the bridging time of filamentous bacteria was not influenced by an increase in the flow factor (Figure 4.5 c, red bars).

In our model, $P_{\text{filament}}$ defines the probability of a dividing bacterium to form a filament and was set to 0.5 for all simulations mimicking filamenting conditions. This probability reflects the amount of filaments in a bacterial population. To determine the influence of a variation in the filamentation probability, the parameter $P_{\text{filament}}$ was set to 0.01, 0.03 and 0.1 and compared with non-filamenting conditions ($P_{\text{filament}} = 0$) and the filamenting conditions used before ($P_{\text{filament}} = 0.5$) (Figure 4.5 d). The model predicts that a probability for filamentation of 0.03 is sufficient to reproduce the effect of filamentation on bridging times that we observed experimentally (Figure 4.3). Higher probability of filamentation did not decrease the bridging time any further (Figure 4.5 d).

To investigate the effect of bridging time on surface colonization, we extended the simulations towards later time points and larger spot arrays. We
4.4 Results

Figure 4.5: Monte Carlo simulations of bacterial bridging of non-adhesive regions. (a) Bridging kinetics of non-adhesive regions for filamentous (red) and non-filamentous conditions (blue). The fraction of the bridged distance ($d_{\text{bridged}}/d_{\text{gap}}$) is plotted over time. The distance between two adhesive spots is bridged three times faster if filamentation is allowed. Representative time series for 20 µm spot distances and flow factor 100 are shown in SI movies S7 (non-filamenting) and S8 (filamenting). (b) Predicted bridging time for spot distances of 5, 10, 20, 40 and 80 µm for filamentous (red) and non-filamentous (blue) conditions. (c) Predicted bridging time of filamentous and non-filamentous bacteria for 20 µm distance at different flow magnitudes. (d) Predicted bridging time of 20 µm distances for different probabilities of filamentation ($P_{\text{filament}}$). For (b), (c) and (d) means and standard deviations of $n=10$ simulations are shown.

modelled a 400 times 300 µm surface area with 10 µm adhesive spot diameter and 20 µm spot distances (Figure 4.2 a, b). Bacterial adhesion on five adhesive spots at the upstream side of the surface was defined as initial condition (Figure 4.6 a, 0 h). The model parameters were set to $P_{\text{filament}} = 0.5$ (filamenting condition), $P_{\text{filament}} = 0$ (non-filamenting conditions) and flow factor $F = 100$ (Figure 4.6 b). As suggested from the previous comparison of filamentous and non-filamentous E. coli surface colonization (Figure 4.3), the filamenting conditions accelerated the surface coverage (Figure 4.6 a).

Finally, and analogous to the experimental setting where we removed cephalixin from the medium, the filament division in the model was resumed after the first 5 doubling periods by setting the parameters $P_{\text{filament}}$ to 0 and $P_{\text{divide}}$ to 0.5 (Figure 4.6). The model again predicts a kinetic advantage of
the filaments over non-filamentous bacteria. This kinetic advantage resulted from the ability of the filaments to bridge the distances to downstream adhesive spots that served as nucleation sites for further surface colonization. By lateral cell-cell contacts, non-filamentous bacteria bridged the 20 µm distance between adhesive spots at later time points (Figure 4.6 a). Those differences in the early colonization kinetics are enhanced at later time points (Figure 4.6 b). We investigated the kinetic advantage of filamentation not only in terms of colonized surface area but also as a function of the number of adhesive spots that were colonized (Figure 4.6 c). Although the filamenting conditions were restricted to the first 5 doubling periods, this was sufficient to colonize two times as many spots than with non-filamenting conditions.

4.5 Discussion

Filamentation is a common trait of various bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella enterica*, and occurs in many natural and industrial habitats [17, 21]. Here we have shown that filamentation of *E. coli* can accelerate the colonization of non-adhesive surfaces that expose microscale adhesive spots (Figure 4.2), mimicking for example defects in a surface coating or heterogeneities within mucosal layers. Non-filamentous *E. coli* were typically washed off by the medium flow when they grew beyond the border of the adhesive spots (Figure 4.3 a, b). In contrast, no offspring is washed away during filament growth. As a result, filamentous *E. coli* bridged non-adhesive distances that are greater than the length of an *E. coli* bacterium shortly before division (4-8 µm) significantly faster (Figure 4.3) although the time needed to double in length is very similar for both phenotypes (25.4 ± 1.6 minutes and 27.6 ± 3.4 minutes, respectively) (Figure S 4.8).

As the microcolonies grew, we observed that non-filamentous *E. coli* were also able to bridge large non-adhesive distances (Figure 4.2 a, b). Since the PLL-g-PEG chemistry used here to passivate the inter-spot areas completely suppressed bacterial binding under low and high flow for at least 9 hours (Figure S 4.7)[9, 10, 31], the bridging of the non-adhesive regions might be possible only by lateral cell-cell interactions. This interpretation is in agreement with the observation that bacteria growing on adhesive surfaces arrange to maximize lateral cell-cell contacts [17]. In the presence of adjacent neighbours within a microcolony, bacteria reached into the non-adhesive area (Figure 4.3 a, bottom panel and b, blue lines). Stabilized by sufficient cell-cell interactions (Figure 4.3 b, 2 h), bridging of the non-adhesive regions can occur gradually.

We implemented a 2D Monte Carlo simulation based on four rules derived from our experimental data. We found good agreement of bridging rates between simulations (Figure 4.5) and experimental data (Figure 4.3). The
4.5 Discussion

Figure 4.6: Filamentation accelerates the bacterial surface colonization of heterogeneous surfaces as shown by Monte Carlo Simulations. (a) Time series of the simulated bacterial colonization of a surface with adhesive spots (green, 10 μm diameter, 20 μm distance) embedded in a non-adhesive surface (white) for non-filamenting (upper panel, blue) and filamenting (lower panel, red) conditions. Until 2:09 h, filamentation was allowed resulting in the formation of filaments (red) that bridge the non-adhesive regions (lower panel, 1:50 h, red filaments). After 2:09 h, the fragmentation of the filaments was resumed by setting $P_{\text{filament}}$ to zero thereby mimicking for example the withdrawal of antibiotics. Further division events resulted in non-filamenting offspring shown in blue (lower panel, 3:40 h). The switch from filamentation ($P_{\text{filament}}=0.5$) to no filamentation ($P_{\text{filament}}=0$) is indicated. The complete time series for (a) are shown in SI movies S9 and S10. (b) Simulated bacterial surface coverage expressed as fraction of the entire area ($400 \times 300 \mu m$) for initial filamenting conditions (red, $P_{\text{filament}}=0.5$) during the first five doubling times. Shown are mean and standard deviation of 3 simulations. (c) The number of adhesive spots (green in Figure 4.6 a) that are colonized is plotted over time. The arrow shows the switch from filamenting (red) to non-filamenting (blue) conditions. Shown are means and standard deviations of 3 simulations. The arrows indicate the switch from filamenting to non-filamenting conditions.
model furthermore allowed us to predict the impact of filamentation on the surface colonization speed and bridging times for conditions not addressed experimentally. Even for adhesive spot distances of 40 and 80 µm, filamentation continued to accelerate the surface colonization (Figure 4.5 b). An higher flow factor $F$ prolonged the bridging time for the non-filamenting system which reflects the influence of increased flow rates and wall shear stresses. Interestingly, changing the flow factor $F$ had no effect under filamenting conditions (Figure 4.5 c). This implies that the prevention of offspring wash-off is the mechanism leading to the observed accelerated bridging between adhesive spots by filaments. Our data suggests that short periods of filament-promoting conditions are sufficient to create a kinetic advantage over non-filamenting bacterial populations (Figure 4.6 b, c). We further varied the probability for filamentation in the model. This reflects the fraction of bacteria in a population that grow into filaments. Our model predicts a threshold probability of 0.03 at which the full kinetic advantage of filamentation is reached (Figure 4.5 d). This suggests that it is sufficient if only a fraction of a bacterial population grows in filaments to have a colonization advantage over non-filamenting bacteria. An increase of the fraction of filamenting bacteria beyond this threshold does not further accelerate the surface colonization.

During the treatment of infections with antibiotics, little attention has been given to the fact that antibiotic doses that are too small to instantaneously kill bacteria might induce their filamentation. Previously, we showed that filamentation of *E. coli* slows their uptake by macrophages [18]. Macrophages have to reach the terminal ends of the filaments before they can form a phagocytic cup and internalize them. Otherwise, they are often observed to contact filaments, even pull on them, but the filamentation in those cases prevents phagocytosis thus impairing an immune response. Here we illustrate a second mechanism where filamentation might have an adverse effect in the fight against biofilms. We show that the antibiotic cephalexin can drastically accelerate the bridging of non-adhesive areas (Figure 4.3). Our results imply that antibiotics that induce filamentation can lead to an accelerated colonization of a heterogeneously adhesive surface, which was not considered in the literature so far and should be taken into account to tailor the dosage of antibiotics. We show that environmental changes that promote a switch in the bacterial phenotype towards filamentation can accelerate bridging of non-adhesive areas (Figure 4.3). We demonstrated that filaments can attach to downstream adhesive regions leaving offspring there, which itself divide and grow into a colony, if the surrounding conditions allow division (Figure 4.4 a). This may also suggest that heterogeneous surfaces in body tissues like Peyer’s patches in the intestine or implant surfaces can be colonized faster if an infection is treated with a filament inducing antibiotic which thus might be detrimental for the host organism.
4.6 Conclusion

Using *E. coli* as bacterial model organism, our study highlights the profound kinetic advantage of bacterial filamentation to accelerate the colonization of heterogeneously adhesive surfaces. Our combined experimental and computational approaches show for early and late time points that filamentation can accelerate the surface colonization. Filamentation increased the rate of bridging non-adhesive areas and can thereby accelerate biofouling of passivated surfaces. While great awareness exists that the build-up of antibiotic resistance serves as intrinsic survival strategy, the local underdosing of antibiotics can switch bacteria into a filamentous state which can accelerate the formation of difficult-to-treat biofilms on heterogeneously adhesive surfaces.

Acknowledgments

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Supplementary information

**Computational model**

*Geometry and initial conditions.* The substrate is described as a 2D lattice, where each lattice site *s*<sub>ij</sub> can be either adhesive (*s*<sub>ij</sub>=1) or nonadhesive (*s*<sub>ij</sub>=0). The bacterial colony is described as a superimposed lattice of the same size, where each lattice site *c*<sub>ij</sub> is either occupied by a single bacterium (*c*<sub>ij</sub>=1), by a part of a filament (*c*<sub>ij</sub>=2), or empty (*c*<sub>ij</sub>=0). For the bridging simulations, the substrate had a size of 50 × 100 with two round adhesive spots of diameter 10 and edge-to-edge distance of 20. The initial conditions were defined as five isolated bacteria adhering to the lower of the two adhesive spots. For the large area simulations, the surface had a size of 280 × 390 with a hexagonal array of adhesive spots of diameter 10 and an edge-to-edge distance of 19, to minimize rounding errors on the discrete lattice. Here, the
initial conditions were defined such that five adhesive spots in the second row
from the bottom were colonized by five isolated bacteria each and separated
by one uncolonized spot each.

Growth without filamentation. In each simulation step, one occupied lattice
site in the colony is selected and its direction of growth is chosen randomly
as one of the four lattice directions (no diagonal growth). The selected cell
is duplicated by shifting the part of the colony in the direction of growth by
one lattice site, so that as a result the number of occupied lattice sites is
increased by one. The duration of the simulation step in units of doubling
time is defined as \(1/n\), where \(n\) = number of occupied sites at the previous
time step. This way, the entire colony has doubled its size on average after
one doubling period. The following rules were implemented:

Filamentation. When a single bacterium is selected to divide, it turns into
a filament with a probability \(P_{filament}\), implemented by changing the value
of the selected lattice site and its neighbor in direction of growth from cell
(1) to filament (2). Filaments can only grow along their long axis. When
a filament grows, it divides with a probability \(P_{divide}\). Filament division is
implemented by changing the value of the selected lattice site on the filament
from 2 (filament) to 1 (single cell), leaving behind two filament fragments.

Surface Detachment. Single bacteria (but not filaments) at the edge of
the colony that are not on an adhesive area are washed away by flow with
a probability \(P_{wash-off}\). The flow direction is always set from bottom to
top in the x-y surface plane. Edge cells at the left and right side of the
colony that have no top and bottom neighbours are therefore more likely to
detach than edge cells at the sides facing the flow direction which have no left
and right neighbors This asymmetry in the wash-off probability leads to an
asymmetric shape of the colony under flow and is implemented in the model by
an anisotropy factor \(A\), which was set to 1/5 for cells facing the flow and 1 for
side-edge cells in order to reproduce the observed colony shapes. Higher flow
magnitude leads to higher wash-off probability. Since the relationship between
flow rate and detachment probability is not exactly known, we describe the
effect of flow magnitude on \(P_{wash-off}\) by introducing a unitless flow factor
\(F\). In every simulation step, all edge cells are identified and removed with a
probability \(P_{wash-off} \leq 1\) that is proportional to \(A\) and \(F\), multiplied by the
duration \(\Delta t\) of the current timestep:

\[
P_{wash-off} = \max(A \cdot F\Delta t, 1)
\]
4.6 Conclusion

Supplementary figures

**Figure S 4.7:** Type 1 fimbriated *E. coli* adhering to differently patterned RNaseB surfaces after 9 hours of surface colonization under 2.4 mL/min flow. The bacterial surface colonization was strongly dependent on the geometries of the micropatterns, which consisted of 10 μm round adhesive spots with spot-to-spot distances of (a) 5 μm; (b) 10 μm and (c) 20 μm. Even after 9 hours under high flow, no bacteria were observed to adhere to the PLL-g-PEG passivation regions for the patterns with 10 and 20 μm distance indicating that the passivation was stable over time.
Figure S 4.8: Comparison of the length doubling time for non-filamentous and filamentous *E. coli* as measured from time-lapse movies. For filamentous *E. coli*, the pole-to-pole length of filaments was measured every 3 minutes over 2 hours. For non-filamentous *E. coli* the pole-to-pole lengths of all the offspring of an individual bacterium were measured every 3 minutes for 2 hours and summed up. The length doubling time was calculated from an exponential fit of the pole-to-pole lengths. Shown are the mean and standard deviations (n=10). Two-independent sample two-sided t-test was performed (α=0.05) and no significant difference (n.s.) was found between the length doubling times. Population variances were not significantly different as tested by a two-sided F-test (α=0.05).
References


References


References


5 Biofilm matrix rescues quorum sensing under flow

In the course of this work, a collaboration was started with Roman Stocker from the Department of Civil and Environmental Engineering at the Massachusetts Institute of Technology, Cambridge, USA. Roman Stocker contributed by discussion on the results and by editing the manuscript. The results presented in chapter 5 were submitted to PNAS. P. Emge, J. Möller and V. Vogel designed the research. P. Emge performed the experiments and analyzed the data. P. Emge, J. Möller R. Stocker and V. Vogel wrote and discussed the manuscript.

5.1 Abstract

Bacteria preferentially grow as surface attached, matrix enclosed biofilms, which account for a large part of chronic bacterial infections. Bacteria in the biofilm are encased in a matrix of extracellular polymeric substances (EPS), which protect cells from environmental stresses, conferring them greatly enhanced tolerance against biocides relative to planktonic cells. Gene expression in biofilm bacteria is often regulated according to the cell density by quorum sensing (QS), an interbacterial communication based on the release and detection of specific inducer molecules. QS is traditionally studied under quiescent conditions, yet biofilms often form in the presence of fluid flow. This results in a competition between fluid flow, which is expected to wash away and thereby reduce the concentration of QS signaling molecules in the biofilm, and the biofilm matrix, which may help retain signaling molecules and thus rescue QS. Here we compared QS under fluid flow in the presence and absence of the biofilm matrix by optically monitoring the onset of QS in Pseudomonas aeruginosa and Escherichia coli under carefully controlled microfluidic flows. We show that in the EPS-producing P. aeruginosa QS also occurs in flow but in the non-EPS-producing E. coli QS is prevented by flow. These findings suggest that, in addition to protecting a population, EPS favors inter-cellular communication within the population by providing a confined habitat in which signaling molecules are retained even in moving fluids, enabling a crucial step in biofilm development under a considerably broader range of environmental conditions.
5.2 Introduction

Cells within bacterial communities often communicate in a population-dependent manner by quorum sensing (QS) [1], a process based on the synthesis, release and detection of small inducer molecules [2]. Various QS systems exist of which the best studied is the LuxI/R-type QS in gram-negative bacteria such as the clinically relevant Pseudomonas aeruginosa [3]. The LuxI-type proteins synthesize acyl-homoserine lactone (HSL) inducer molecules that are released by the bacteria into the surrounding fluid. In the absence of fluid flow, the local HSL concentration increases with the bacterial population density [4]. Above a threshold concentration, the inducer molecules bind to their cognate LuxR-type receptors. The inducer-receptor complex then targets the expression of a set of genes, including genes involved in virulence and resistance [5, 6].

Most biofilm habitats are not quiescent, and are instead characterized by ambient fluid flow, which is expected to wash out signaling molecules from the bacterial community, thereby lowering local concentrations below the threshold required for QS [4, 7]. Yet, QS is known to occur in environments where flow is present, such as rivers and streams in nature or catheters in clinical settings [8]. One mechanism by which signaling molecules can accumulate to sufficient concentrations even under flow may be the production of a matrix of extracellular polymeric substances (EPS) secreted by biofilm bacteria [9, 10]. This self-produced network of polysaccharides, proteins, DNA and lipids may prevent or delay the washout of QS inducer molecules by the external flow and thus broaden the ambient conditions under which QS can occur, which has not been addressed before in spite its broad implications for biofilms in a wide range of environments.

To investigate the potential sequestering function of the EPS matrix and the influence of external fluid flow on bacterial QS, we study the role of EPS matrix production in P. aeruginosa QS-dependent gene expression. We compare the expression of a QS-controlled GFP reporter under fluid flow in the presence and absence of the EPS matrix, to test the hypothesis that fluid flow prevents the onset of QS in the absence of EPS, but enables it in the presence of EPS. Because of the virtual impossibility of completely removing from a P. aeruginosa biofilm the EPS matrix [9], which is necessary to prevent detachment of bacteria under flow [11], we also performed experiments with the same QS system expressed in E. coli K-12, which does not produce significant amounts of EPS [12]. Genetically engineered QS systems in E. coli are widely applied approaches in QS research [13–15]. Here, we expressed the Lux-type QS system LasI/R [3] from P. aeruginosa recombinantly in E. coli by engineering and expressing a synthetic QS circuit composed of lasR under the control of a constitutive promoter as well as lasI and gfp under the
control of the QS controlled \textit{las}-promoter. Our synthetic QS system controls the expression of the green fluorescent protein (GFP) and thus allows for real-time optical monitoring of the onset of QS. By expressing \textit{P. aeruginosa}'s innate \textit{LasI}/\textit{R} system in \textit{E. coli} we can study the role of EPS production in \textit{P. aeruginosa} without the need of a \textit{P. aeruginosa} “matrix knock-out”. For \textit{P. aeruginosa}, we use a chromosomal GFP fusion to optically monitor the QS controlled \textit{rhlA} expression [16]. EPS matrix production was monitored by adding to the growth medium the fluorescently labeled lectin concanavalin A (conA), which specifically binds mannose residues, a major component of the polysaccharides in \textit{P. aeruginosa}’s matrix [17].

Comparing the QS-dependent expression of an optical GFP reporter between two systems – \textit{P. aeruginosa} and \textit{E. coli} – which widely differ in EPS production revealed that, while independent of matrix production under quiescent conditions, the onset of QS in the presence of fluid flow requires an EPS matrix, which rescues QS under flow conditions. By using microfluidic flow channels we can carefully control the flow and convection applied to a bacterial community and in combination with optical reporters assess its influence on the onset of QS.

5.3 Materials and methods

5.3.1 Bacterial strains and media

Bacterial cultures were grown from glycerol stocks in lysogeny broth (LB) medium [18] with appropriate antibiotic (100 µg/mL ampicillin; 50 µg/mL kanamycin; 10 µg/mL chloramphenicol) at 37 °C in a shaking incubator (Infors HT) over night. LB medium with appropriate antibiotics was inoculated with these cultures and grown to the desired optical density (OD$_{600}$) for the experiments.

5.3.2 Construction of quorum sensing circuits

The quorum sensing (QS) circuits for the expression of the RhlI/R and LasI/R systems in \textit{E. coli} were constructed from plasmids kindly provided by F. Arnold [13]. \textit{lasI} was PCR amplified using oligo sp\textunderscore lasI-502-BamHI (5’-AAA-AGGATCCAAAGAGGAGAAATTAAGCAT-3’) and oligo asp\textunderscore lasI-502-KpnI (5’-AAAAGGATCCAAAGAGGAGAAATTAAGCAT-3’). The PCR product was digested with BamHI and KpnI and cloned into the BamHI and KpnI sites of plasmid pFNK-503-qscrsaL, resulting in plasmid pMG401 (Figure 5.1 a). The backbones were dephosphorylated by shrimp alkaline phosphatase prior to ligation. The sequence integrity of the construct was verified by sequencing purified plasmids at Microsynth AG. All PCR reactions were carried out in
a thermocycler (Eppendorf mastercycler) following standard protocols [19]. Primers were ordered at Microsynth AG (Balgach, Switzerland). Enzymes were purchased from New England Biolabs.

5.3.3 QS reporter validation of the constructed circuits on solid agar medium

The functionality of the constructed circuits and the \textit{P. aeruginosa} QS reporter was confirmed by growing \textit{P. aeruginosa} QSR and \textit{E. coli} QSR on LB agar medium containing the appropriate antibiotic. The cells were streaked from glycerol stocks onto LB agar petri dishes and incubated at 37 °C over night. A single colony was picked and resuspended in 50 µL PBS. The suspension was washed once in 50 µL PBS and resuspended in 10 µL PBS. This suspension was analyzed by microscopy.

The dose response was measured in batch cultures (\textit{E. coli} ctrl) or in individual surface attached bacteria (\textit{P. aeruginosa} QSR). \textit{E. coli} ctrl was grown in LB medium to a OD\textsubscript{600} of 0.2. 200 µL of this cell suspension was added to 100 µL LB containing a serial dilution of C12-HSL (100 nM – 0.01 nM) or only LB (0 nM) in a black 96 well plate with optical bottom (Nunclon). The plate was incubated at 37 °C for 1.5 h before fluorescence intensity was measured with a plate reader (Tecan Infinite M200).

\textit{P. aeruginosa} QSR were grown to an OD\textsubscript{600} of 0.06 and used to seed a flow chamber. LB medium containing first no and then ascending concentrations (0.01-10\textsuperscript{3} nM) C12-HSL was applied to the surface attached cells with a syringe pump. At each concentration, the system was incubated for 60 minutes with continuous flow before image acquisition.

5.3.4 Flow chamber assays

All flow chamber assays were carried out in polydimethylsiloxane (PDMS) flow chambers. PDMS was molded from a microfabricated SU8 master on a silicon wafer. The flow channel dimensions were 1 mm width, 40 µm height and 3 cm length and a syringe pump (Harvard Apparatus PHD ultra) was used to apply flow at 0.6 µL/min. For matrix visualization, 20 µg/mL concanavalin A (conA), tetramethylrhodamine conjugate (Invitrogen C860) was added to the flow medium.

For \textit{E. coli}, the flow chamber glass bottom was coated with 20 µg/mL RNaseB in 0.02% bicarbonate buffer for 30 minutes at room temperature. For \textit{P. aeruginosa}, the flow chamber glass bottom was incubated with 20 µg/mL poly-L-lysine (PLL) likewise. To seed the flow chambers, bacterial cultures in the log phase (OD\textsubscript{600} = 0.4-0.6) were washed once in PBS and resuspended in PBS to a final OD\textsubscript{600} of 0.02.
5.3.5 Microscopy techniques

All microscopic data was acquired by phase or differential interference contrast (DIC) microscopy (Nikon TE2000-E) with a 60× oil immersion objective. Fluorescence images were acquired by fluorescein isothiocyanate (chroma 49002) and tetramethylrhodamine (chroma 49005) filter sets. Images were acquired with an electron multiplying charge-coupled device (EM-CCD) camera (Hamamatsu C9100-02).

5.4 Results

Biofilms were grown on the bottom glass slide of microfluidic channels for 23 hours under carefully controlled flow conditions. The kinetics of QS onset for all bacterial strains was studied optically by continuously monitoring the increase of GFP fluorescence that is expressed recombinantly under QS control. For experiments in flow conditions, a flow rate of 0.6 µL/min (mean flow velocity = 0.25 mm/s; wall shear stress = 0.0034 pN/µm²) was applied. For *E. coli*, which do not form a matrix, adhesion under flow was ensured by coating the glass slide with the mannose-presenting protein RNaseB, to which *E. coli* firmly adhere by the adhesin FinH on their type 1 fimbriae [20]. For *P. aeruginosa*, initial adhesion was promoted by coating the glass slide with PLL. All experiments were performed in LB medium at 37 °C.

5.4.1 Characterization of the engineered quorum sensing reporter strains and the dose response of the HSL inducer molecules

To study the effect of the EPS matrix on the accumulation of QS inducer molecules, we compared matrix producing *P. aeruginosa* PAO1 with non-matrix producing *E. coli* K-12. We engineered a plasmid-encoded construct and expressed it in *E. coli*, where *lasI* and *gfp* are under the control of the QS controlled promoter p(*las*) and *lasR* is constitutively expressed from the p(*laqI*) promoter (*E. coli* QSR, Figure 5.1 a). LasR synthesizes 3-oxo-C_{12} HSL (C12-HSL), which binds to its cognate receptor LasR. The LasR-C12-HSL complex then promotes expression of GFP. The negative control is represented by the same construct but without *lasI* (*E. coli* ctrl, Figure 5.1 a).

In *P. aeruginosa* wild type strains, the expression of RhlA as a marker for QS is promoted by the LasI/R and RhlI/R systems, and the RhlI/R system activation depends itself on LasI/R (Figure S 5.5). A translational *rhlA::gfp* fusion allows for in situ optical monitoring of QS onset by epifluorescence microscopy [16]. Once the QS controlled *rhlA* transcription is activated, the bacteria co-express GFP, which results in green fluorescent *P. aeruginosa*. All plasmids and strains are listed in Table 5.1.
Figure 5.1: Characterization of reporter constructs for QS. (a) To obtain *E. coli* QSR, the *P. aeruginosa* LasI/R QS reporter system is expressed from an engineered plasmid. (b) Dose response curves, obtained from GFP fluorescence of individual surface-attached cells (*P. aeruginosa* QSR; 10 cells) and for a batch culture (*E. coli* ctrl; triplicate), for different concentrations of the inducer C12-HSL added to the medium. Errors bars denote standard deviations. (c) DIC and GFP fluorescence micrographs of *P. aeruginosa* and *E. coli* grown on solid agar. (d) The fraction of bacteria that showed QS controlled GFP fluorescence for *P. aeruginosa* QSR (n = 2240) and *E. coli* QSR (n = 1388). Errors bars denote standard deviations.
5.4 Results

Table 5.1: Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Description</th>
<th>Designation</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFNK-102</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt; p15A, source of rhlI</td>
<td>[13]</td>
<td></td>
</tr>
<tr>
<td>pFNK-502-RBSII</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt; p15A, source of lasI</td>
<td>[13]</td>
<td></td>
</tr>
<tr>
<td>pFNK-202-qec119</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt; p15A containing rhlR and gfp</td>
<td>[13]</td>
<td></td>
</tr>
<tr>
<td>pFNK-503-qecrsL</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt; p15A containing lasR and gfp</td>
<td>E. coli ctrl</td>
<td>[13]</td>
</tr>
<tr>
<td>pMG401</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt; p15A containing rhlR, rhlI and gfp</td>
<td>E. coli QSR</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1</td>
<td>P. aeruginosa wild type</td>
<td>P. aeruginosa wt</td>
<td>DSMZ&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>SMC01201</td>
<td>Gen&lt;sup&gt;r&lt;/sup&gt; P. aeruginosa with a chromosomal rhlA::gfp transcriptional fusion</td>
<td>P. aeruginosa QSR</td>
<td>Gift from G. O’Toole</td>
</tr>
<tr>
<td>MG1655</td>
<td>F- λ- rph-1 E. coli K-12</td>
<td>E. coli QSR</td>
<td>DSMZ&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>http://www.dsmz.de/

To compare if the QS systems of *P. aeruginosa* and *E. coli* respond to the same C12-HSL inducer molecule concentration we acquired a dose response of both systems. To avoid self-activation, the concentration of C12-HSL that activates rhlA expression in *P. aeruginosa* was not measured in batch cultures. Instead, we seeded the bottom glass surface of a microchannel with *P. aeruginosa* in the logarithmic growth phase (OD<sub>600</sub> = 0.06). Micrographs were acquired over time at multiple positions and GFP expression of single cells of *P. aeruginosa* QSR was analyzed to detect QS. At this population density bacteria show no GFP fluorescence (Figure 5.1 b), indicating that the extracellular concentration of self-produced inducer molecules is too low to trigger QS. Upon addition of the C12-HSL inducer to the flow medium, the bacteria started to fluoresce at an HSL concentration of 10 nM. Maximum GFP fluorescence occurred at 100 nM C12-HSL (Figure 5.1 b).

To determine the dose response of the *E. coli* QS reporter constructs, the *E. coli* ctrl strain was used to avoid activation of the QS controlled GFP expression by self-produced inducer molecules. The *E. coli* ctrl strain fluoresces at and above inducer concentrations of 1 nM C12-HSL and fluorescence plateaus at 10 nM C12-HSL (Figure 5.1 b). Similar concentrations (23 nM [7] and 1 µM [21]) have been reported to fully activate LasR regulated genes in lacZ assays for *P. aeruginosa*. Our results indicate that both reporter systems respond to a C12-HSL concentration above 1 nM and thus allow a comparison of the QS system across the two strains.
5.4.2 QS is independent of EPS matrix in agar grown colonies

In quiescent systems we expect a QS dependent gene expression irrespective of the presence of an EPS matrix since the inducer molecules can more readily accumulate in the vicinity of the bacteria, as the only process that dilutes them is molecular diffusion. To verify this prediction, *P. aeruginosa* QSR and *E. coli* QSR were grown overnight on LB agar plates in the absence of external flow, and colonies were analyzed by epifluorescence microscopy. The fraction of bacteria with activated QS was determined by image thresholding and automated counting. 90% of *P. aeruginosa* QSR cells and 98% of *E. coli* QSR cells exhibited QS under these quiescent conditions (Figure 5.1 d). Wild-type *P. aeruginosa* cells, lacking the GFP reporter, showed only minimal (auto)fluorescence and the *E. coli* ctrl cells, lacking lasI, showed no fluorescence (Figure 5.1 c). This indicates that the engineered QS constructs are functionally expressed in *E. coli* and that for both systems – and therefore irrespectively of the presence of an EPS matrix – HSL inducer molecules accumulate to a sufficient concentration to induce QS under quiescent conditions.

5.4.3 QS is independent of EPS matrix in quiescent liquid cultures

The independence of QS from the presence of an EPS matrix, suggested by the agar experiments with agar grown colonies, was confirmed with experiments in quiescent liquid cultures, with no medium exchange. In this case, too, one expects secreted inducer molecules to progressively accumulate in the medium and the QS GFP reporter systems to be activated irrespective of the presence of a matrix. To test this hypothesis, *P. aeruginosa* QSR and *E. coli* QSR were grown as liquid cultures in 96-well plates, without stirring, and changes in GFP fluorescence were measured with a plate reader. The fluorescence of *P. aeruginosa* QSR increased after 9 h of growth and plateaued at approx. 12 h (Figure 5.2 a). This signal originated from the QS controlled GFP expression, not from autofluorescence, as confirmed by the lack of fluorescence from *P. aeruginosa* wt (Figure 5.2 a). The fluorescence of *E. coli* QSR increased after 3 h of growth and also plateaued at approx. 12 h (Figure 5.2 a). No fluorescence was observed for *E. coli* ctrl (Figure 5.2 a), which does not produce C12-HSL.

To eliminate the effect of different initial seeding densities in these liquid cultures (Figure 5.2 a), we determined the dependence of the fluorescence intensity on the population density, obtained by absorbance measurements (turbidity) at 600 nm (Figure 5.2 b). *P. aeruginosa* QSR starts to fluoresce at a turbidity of approx. 0.5 (Figure 5.2 b), whereas *P. aeruginosa* wt shows no fluorescence at any turbidity (Figure 5.2 b). For *E. coli* QSR, fluorescence was detected from the start (Figure 5.2 b), which may in principle
5.4 Results

Figure 5.2: Quorum sensing in quiescent liquid cultures occurs both in the presence and absence of a matrix. (a) Time series of GFP fluorescence measured in 96-well plates for the QS reporter strains and the appropriate control strains. The absolute intensity of fluorescence in the *E. coli* QSR strain is substantially higher compared to *P. aeruginosa* QSR which might be due to the synthetic ribosomal binding site (RBS) II upstream of the lasI and lasR insertion. (b) The same data, plotted as a function of bacterial population density, measured as the turbidity of the liquid culture at 600 nm. Error bars denote standard deviations.

suggest a constitutive expression of GFP. Lack of fluorescence from *E. coli* ctrl for all turbidities, however, rules out this possibility and confirms that GFP expression in *E. coli* QSR is QS dependent but the system is highly sensitive. Together, results from agar colonies (Figure 5.1 c) and liquid cultures (Figure 5.2) demonstrate the activation of QS in both reporter strains, *P. aeruginosa* QSR and *E. coli* QSR, under quiescent conditions.

5.4.4 *P. aeruginosa* is able to quorum sense under flow

To test if ambient fluid flow affects the onset of QS, the two QS reporter strains were grown in microfluidic channels (1 mm x 40 µm cross section) in the presence of a constant, physiologically relevant 0.6 µL/min flow of medium, corresponding to a mean flow speed of 25 mm/s and wall shear stress of 0.034 dynes/ cm². Physiological shear stresses span a wide range, from 0.0001-0.4 dynes/cm² in interstitial compartments [22], to 1 dyne/cm² in saliva and 30-40 dynes/cm² in blood [23]. Bacterial growth on the bottom glass surface of the microchannel was monitored by differential interference contrast (DIC) microscopy and the fluorescence of QS dependent GFP expression by epifluorescence microscopy. Starting from a low initial seeding density on the surface, the population density of *P. aeruginosa* QSR increased until bacteria were densely packed (Figure 5.3 a). The GFP signal increases only slightly until 16 h of growth, when a steep increase in GFP fluorescence is observed (Figure 5.3 c, e), marking the onset of QS. The effect of autofluorescence on this signal is minimal, as confirmed by comparison with *P. aeruginosa* wt (Figure 5.3 c, Figure S 5.6).
This observation is in marked contrast with results for *E. coli* QSR, which shows no GFP signal under fluid flow for the 23 h duration of the experiments (Figure 5.3 d, f), suggesting that fluid flow prevented the concentration of inducer molecules to reach the threshold for activation of QS. *E. coli* QSR, too, grew from a low initial seeding density to a closely packed colony on the surface (Figure 5.3 b), with a density comparable to that of *P. aeruginosa* QSR (Figure 5.3 a). Therefore, the differential expression of QS in the two reporter strains cannot be attributed to different population densities, and suggests instead a critical role of the EPS matrix in preventing the washout of inducer molecules by flow.

The hypothesis that the EPS matrix enables QS in flowing fluid predicts a temporal correlation between QS onset and matrix production in *P. aeruginosa* QSR, whereby QS can begin only after the matrix has been produced. This prediction was confirmed by monitoring the production of EPS through the addition of a rhodamine labeled lectin (conA) to the flowing medium. ConA binds to α-D-mannopyranosyl of the mannose-rich polysaccharide products of the *psl*-operon, which represent a considerable fraction of the EPS of *P. aeruginosa* [17, 24, 25]. For *P. aeruginosa* QSR, conA fluorescence increased considerably after 8 h of growth (Figure 5.3 c), indicating that the matrix was built several hours before QS became prominent (approx. 4 h; Figure 5.3 c). The EPS matrix forms a heterogeneous, fiber-like network structure (Figure 5.3 g), similar to that previously observed for *P. aeruginosa* grown as pellicles [24]. No increase in conA fluorescence was detected for the 23 h of the experiments when conA was added to *E. coli* QSR (Figure 5.3 d, f), indicating the absence of mannose-rich sugars, in line with the known lack of significant EPS production in *E. coli* [12]. The initially higher conA signal in Figure 5.3 d is probably due to binding of conA to mannose presented on RNaseB (see supplementary information).

Taken together, these findings suggest that the absence of the *P. aeruginosa* EPS in combination with medium flow can inhibit the accumulation of the C12-HSL molecule to threshold concentrations and thus prevent QS.

### 5.4.5 Addition of exogenous inducer molecule allows the *E. coli* QS reporter strain to quorum sense under flow

To further support the hypothesis that the lack of QS in *E. coli* QSR in the presence of fluid flow (Figure 5.3 d, f) is due to insufficient accumulation of C12-HSL inducer molecules, we repeated the microfluidic experiments and added after 21 h 10 µM C12-HSL to the flowing medium, a concentration sufficient to produce full activation in quiescent conditions (Figure 5.1 b). Thirty minutes after C12-HSL addition, *E. coli* QSR exhibited bright GFP fluorescence (Figure 5.4), demonstrating that the QS reporter system is fully
5.4 Results

Figure 5.3: Fluid flow suppresses QS in *E. coli* QSR, but not in *P. aeruginosa* QSR. Both strains were grown in a microfluidic channel under continuous fluid flow. (a, b) DIC images of *P. aeruginosa* QSR and *E. coli* QSR, showing the time course of surface coverage. (c) Time series of QS dependent GFP fluorescence intensity and matrix production for *P. aeruginosa* QSR; the increase in fluorescence of *P. aeruginosa* wt is due to autofluorescence (also see Figure S 5.6); the matrix production is visualized by staining the Psl EPS with conA. Due to the high population density (see panel A), single cells are not resolved in epifluorescent images. (d) Time series of QS dependent GFP fluorescence and matrix production in *E. coli* QSR. Shown are mean grey values of 10 images taken at different locations in the microchannel. Errors bars are standard deviations of the mean. (e, f) Image series of the data shown in c and d. Only parts of the images analyzed are shown. (g) Confocal image of the conA-stained matrix of *P. aeruginosa* QSR.
5 Biofilm matrix rescues quorum sensing under flow

Figure 5.4: Addition of exogenous inducer molecule induces GFP expression in E. coli QSR also under flow. Thirty minutes after addition of 10 µM C12-HSL, which occurred at time 21:00 h, E. coli QSR shows strong QS dependent GFP expression in the presence of fluid flow.

functional also under fluid flow and strongly suggesting that the failure to produce a GFP signal by self-induction is due to an insufficient accumulation of inducer molecule.

5.5 Discussion

Over the last decade, several functions of the matrix that encases biofilm bacteria have been understood. The EPS matrix confers bacteria in biofilms enhanced resistance to antibiotics, disinfecting agents and grazing predators compared to their planktonic counterparts [26–29], in addition to a mechanism for strong surface adhesion [9]. Our results demonstrate a new function of the EPS matrix, by showing that the matrix can rescue QS when there is external fluid flow, most likely by preventing the wash-out of inducer molecules. The EPS matrix consists to 98% of water, which is essential to keep the resident bacteria from dehydrating, and supports a porous network of channels within the biofilm that helps irrigate the deeper locales of the biofilm, supplying cells with nutrients and ridding them of waste products [30]. Our results show that, under quiescent conditions, QS becomes activated irrespective of the presence of a matrix (Figure 5.1, 5.2), whereas under ambient flow, the occurrence of QS is dependent on the presence of an established matrix (Figure 5.3 c, d).

The mechanism by which the EPS matrix enables QS in flowing environments may be explained by a sequestering effect, by which the matrix may prevent the dilution of QS inducer molecules by hampering both diffusion and convection. The EPS matrix can considerably reduce and retard the diffusion of small molecules [31, 32] and, being charged and hydrophobic, can interact with and potentially retain cationic and hydrophobic compounds [30, 32, 33]. This may contribute to explain the enhanced tolerance of biofilms against antimicrobial agents [31, 32, 34], which can become immobilized within the EPS matrix by electrostatic interactions [33]. In line with this evidence, our results suggest that the EPS matrix effectively traps the autoinducer molecules responsible for QS, enabling them to accumulate to sufficient concentrations to
induce QS when fluid flow would otherwise wash them out. Charlton reported
an accumulation of QS inducer molecules in biofilms compared to the super-
fluent medium [35], however, it was unclear if the QS molecules found were
from within the bacterial cells or retained in the matrix.

Supporting this result is the observation that the EPS matrix forms prior
to the onset of QS (Figure 5.3 c). This suggests that the psl-operon that
controls the expression of the conA-stained Psl EPS component is regulated
independently of QS. This is in agreement with the finding that the psl-operon
is controlled by a $\sigma^{70}$-promoter and not a QS target [36, 37].

Taken together, these results suggest that the EPS matrix prevents $P.$
$aeruginosa$ inducer molecules from being washed away by flow, thus enabling
QS to be induced also in flowing environments. One possible advantage of
a sequestering effect is that it enables QS in the broad range of microbial
environments characterized by flow, by enhancing the retention of small QS
inducer molecules and thus enabling their concentration to rise in the vicinity
of the bacteria. Flow is common in bacterial habitats, including the urinary
tract, implants and catheter surfaces, as well as natural environments such as
rivers, streams, marine animal skins and the subsoil. In this very broad range
of environments, without retention by the matrix, the QS inducer would be
washed away by the slightest fluid flow and bacterial communities could not
express the QS controlled genes.

The control that QS exerts on virulence factors [6, 38] makes QS a target
for antimicrobial drugs [39]. Beyond employing QS antagonists, interfering
with the matrix’s sequestering effect for QS inducer molecules may provide a
new approach to suppress QS, and thus detrimental effects such as virulence or
biofilm maturation. The mechanism leading to sequestering may be a physical
reduction in the strength of fluid flow in the immediate vicinity of the bacteria
releasing the inducer molecules and hence a lower rate of transport away from
that region. Disrupting the matrix should therefore prevent QS, however, it
also leads to dispersal of the biofilm [11] and does not allow to assess the
role of the EPS in QS. Alternatively, the sequestration could be of chemical
nature but the signaling molecules need to stay available for the bacteria
which would be impaired by covalent binding by the signaling molecules to
the matrix. Weak hydrophobic interactions of signaling molecules and matrix
are also possible and changing the hydrophobicity of the matrix may change
its ability to sequester the inducer molecules.

Although an exact mechanism of signal retention has yet to be found, its
understanding and prevention can offer a new way to interfere with bacterial
QS of biofilms in industrial an clinical settings and thus reduce the persistence
and resistance of the negative implications caused by biofilms.
5 Biofilm matrix rescues quorum sensing under flow

5.6 Conclusion

Biofilms constitute more than 99% of the microbial biomass on earth [40] and occur virtually everywhere, from freshwater and marine environments, to hot springs, deep-sea vents, soils and animal tissues [41, 42]. Biofilms are of great clinical interest as they account for a major part of chronic infections in the human body [27, 28, 31]. Living in a biofilm enhances the antibiotic resistance of the resident cells and offers them protection from toxic agents, dehydration, acid exposure and UV irradiation [28]. Our data shows an additional, fundamental function of the EPS matrix: the matrix allows bacteria to engage in QS also under flow conditions, and lack of a matrix prevents QS. Investigating bacteria under flowing fluid is a more realistic model system for many natural environments compared to quiescent conditions, and our results demonstrate that including fluid flow can lead to macroscopic differences in at least one important biofilm process – quorum sensing – and thus presumably to the processes under the control of QS, including virulence and antibiotic resistance. The ability to rescue QS in flow environments is a before unrecognized function of the biofilm matrix. This work also indicates that a tout-court application to flowing environments of results on the effectiveness of QS antagonists or biocides obtained under quiescent conditions may prove unjustified. Instead, we suggest that screening assays for new approaches for biofilm disruption should explicitly include matrix formation and ambient fluid flow as fundamental parameters, especially if drugs that interfere with QS are investigated.

Acknowledgments

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Supplementary information

*E. coli* did not adhere permanently on the microfluidic glass surface under flow, in line with previous evidence [43] and further supporting *E. coli*’s lack of extensive matrix production. In order to firmly attach *E. coli* QSR in flow, we coated the microfluidic bottom glass surface with RNaseB, to which *E. coli* binds using type 1 pili [20]. Seeding of bacteria occurred in conA-free medium, since both conA and type 1 pili are mannose-specific and may bind competitively. When added to the *E. coli* QSR cultures, no increase in conA fluorescence is detected (Figure 5.3 d), indicating that no mannose-rich sugars are present or secreted during the 23 h experiment. However, the initial fluorescence is twice that of *P. aeruginosa* QSR, possibly due to the glycoconjugates of RNaseB present on the microchannel surface that are stained by the conA.

The GFP fluorescence of the QS activated *P. aeruginosa* QSR was weak and single bacteria were not resolved by wide-field fluorescence microscopy (Figure 5.3 e). To visualize *P. aeruginosa* QSR in fixed samples we added DAPI stain (Figure S 5.7 a). The shown maximum intensity projection indicates the presence and association of single *P. aeruginosa* QSR bacteria with the Psl EPS matrix. A confocal laser scanning micrograph and a 3D reconstruction of the corresponding conA-TRITC stained EPS matrix and DAPI stained bacteria show that the bacteria are homogeneously distributed in the matrix (Figure S 5.7 b).

**Figure S 5.5:** Upper panel; Insertion of the *gfp* gene sequence downstream and in-frame of the *rhlA* gene allows for optical read out of QS onset in *P. aeruginosa*. Lower panel; *P. aeruginosa* wild type (wt). The LasI/R system induces the RhII/R system which in turns induces the *rhl*-operon.
5 Biofilm matrix rescues quorum sensing under flow

Figure S 5.6: Image series to Figure 5.3 e, *P. aeruginosa* wt. Growing *P. aeruginosa* wild type results in only a slight increase of fluorescence which is supposedly autofluorescence.

Figure S 5.7: (a) Confocal laser scanning micrographs of a *P. aeruginosa* QSR biofilm after fixation and addition of DAPI stain. The bacteria are embedded within the conA-stained EPS matrix. (b) 3D representation of *P. aeruginosa* QSR grown in the presence of fluid flow for 27 h, measured using confocal microscopy. The GFP fluorescence (green) indicates expression of the QS controlled *rhlA::gfp* fusion and the mannose-rich structures of the matrix were labeled with conA (red).
References


References


References


References


6 Outlook

Biofilm formation on tissues and engineered surfaces pose a large medical and economical risk. As a consequence, much effort is spent in the prevention of biofilm formation. In the fight against biofilms three major lines of defense can be identified: firstly, killing of bacteria in the first place is the primal and most intuitive approach. Usually biocides such as many antibiotics or antiseptics or sterilization methods aim at killing bacteria by disintegrating membrane integrity, disturbing protein synthesis and cross-linking or precipitate proteins. Where most of these methods are very effective against planktonic bacteria, the biofilm mode of live provides to some extend protection from them. Antiseptics and antibiotics are reported to be by far less effective against biofilms [1] and physical methods such as sterilization by heat or UV irradiation are often not possible because of limited access (biofilms in pipes, on implants in a patient) and even to such radical methods, biofilms show an enhanced resistance [2].

As killing seems not to be a robust approach in all relevant situations and becomes harder as bacteria grow as surface attached biofilms, the second strategy aims at preventing bacterial adhesion to tissues and surfaces. For specific receptor-ligand interactions in tissue adhesion, blocking the bacterial receptor is a promising approach [3]. For unspecific adhesion to engineered surfaces the coating with antimicrobial compounds has proven to drastically reduce initial bacterial adhesion [4]. However, due to instability of and defects in the coating and morphological adaptions of bacteria, only few adherent bacteria are sufficient to result in formation of a biofilm albeit the speed of such a formation is considerably prolonged [5].

If the killing of bacteria fails and the adhesion though reduced still can lead to biofilm formation, a third line of defense is to interfere with physiological changes in bacteria such that virulence development or biofilm stability is impaired. Many of these processes are quorum sensing controlled and a lot of effort is spent in the search of quorum sensing antagonists, which are promising drugs to prevent bacterial pathogenicity [6].

In this thesis, a very specific part of each of these aspects is addressed. First, the presented viability assay allows to gain kinetic insight into the mode of action of surface bound biocides at a cellular level. Nevertheless, it requires genetic engineering of the bacteria to be investigated and adaption of stains to non-toxic levels. The development of a entirely non-invasive assay could
be of interest. An approach to accomplish this may be the exploration of the lifetime of autofluorescence of bacteria. Sensitive fluorescence lifetime imaging (FLIM) can be used to monitor the lifetime of bacterial autofluorescence. Due to loss of membrane integrity when bacteria are killed, the autofluorescence lifetime may be changed and serve as an indicator of bacterial viability.

Second, the study on the kinetics of surface colonization by bacteria revealed filamentation to provide a kinetic advantage in colonizing heterogeneously adhesive surfaces. \( \beta \)-lactam antibiotics can trigger the filamentation. Varying the antibiotic concentrations in a physiologically relevant range, as it occurs in actually treated patients, could add to the model as it allows to gain insight in how the antibiotic influences surface colonization of tissue or implants in patients.

Our third study revealed a role of the biofilm matrix in quorum sensing under flow conditions. Experiments with a mutant \( P. \ aeruginosa \), deficient in HSL production, can lead to a more refined understanding of the role of the matrix, for instance in mixed cultures with wild type \( P. \ aeruginosa \). A systematic investigation on the effect of different flow magnitudes to the onset of quorum sensing may be of interest, since in natural habitats the flow rates can vary greatly.

The habitats in which bacteria naturally occur are characterized by complex environmental factors. The models and tools we provide include some of these environmental parameters such as ambient fluid flow, filamentation, heterogeneity of surfaces, matrix formation and quorum sensing. By including these parameters, bacterial models systems are refined and reflect the natural situation more accurately. This is required to understand physiological processes in bacterial communities and to find new solutions in unanswered questions.

### 6.1 Future directions

The combination of the unimaginably large field of biofilm research with bioengineering offers new possibilities in understanding how bacteria get along in their complex environment. The aspect of fluid flow for instance is far from being exploited. Flow, and thus nutrient availability, can have an influence on the metabolic rate of bacteria [7] and thus influence many processes such as quorum sensing, matrix formation, and gene expression. A systematic exploration of these aspects can yield further parameters for more complex models.

The properties of the substrate to which bacteria adhere and how these influence biofilm formation is a field currently being explored. Electrostatic, chemical and topographic properties are investigated [8] and the substrate
stiffness is of interest, too [9]. However, exact mechanisms of how the surface influences adhesion and biofilm formation remain unresolved for many of the surface properties. It would be interesting to investigate if surface properties can have a direct influence on bacterial signaling and may cause a delay or promotion of quorum sensing onset.

In nature, biofilms often contain more than a single species [10]. Addressing this by investigating multi-species biofilms can further increase the complexity of model systems. Many of the open questions such as how the inherent resistance of biofilms comes about, may only be answered using complex model systems which incorporate effects such as matrix, flow or the effect of different species in the biofilm.

Biofilms provide also protection from uptake by macrophages [2], but after initial adhesion, not yet in a biofilm, macrophages are well able to uptake bacteria by a recently described hook and shovel mechanism [11]. Incorporating macrophage in a model may answer the question when the grazing ability of macrophages comes to a stop. It may well be that this is correlated with matrix production.

6.2 Closing remarks

The field of biofilm research is a sheer limitless one and unanswered questions of high significance and general interest can be found in any direction. A large potential lies probably where engineering meets microbiology, offering the possibility of interdisciplinary research. Finding answers to these questions may help to understand the mechanisms that lead to the detrimental effects that biofilms can have in medical and technical settings, which in turn can help to reduce the negative implications of bacterial biofilm formation.
References


Appendix

1 QS reporter system in *E. coli*

The strain *E. coli* QSR described in chapter 5 contained the plasmid pMG401 (Figure 1 A). *lasR* and the kanamycin resistance cassette are under the control of the constitutive *lacI* promoter [1] and are one transcriptional unit terminated by T0. *lasI* and *gfp* are under control of the quorum sensing controlled (*qsc*) *rsaL* promoter which is controlled by the LasI/R system [2]. *lasI* and *gfp* are a second transcriptional unit terminated by T1. The replication of the plasmid is controlled by the p15A replicator and occurs at approx. 15 copies per cell [3]. *lasR*, *lasI* and *gfp* are translated from a synthetic ribosomal binding site (RBSII). The plasmid pMG401 was cloned from plasmids pFNK-502-RBSII and pFNK-503-qscrsaL which were kindly provided by Prof. Frances Arnold (California Institute of Technology, California, USA) [4].

The C12-HSL/LasR controlled GFP expression in *E. coli* was characterized. Surface attached *E. coli* in a flow chamber were supplied with exogenous C12-HSL and GFP fluorescence was measured over time (Figure 1 B). Five minutes are sufficient to detect GFP expression compared to the control without addition of C12-HSL. To determine the fraction of bacteria that start GFP expression upon C12-HSL addition, the fluorescence images were automatically thresholded and the bacteria were counted (Figure 1 C). After 12 min approx. 85% of the bacteria are activated and no further increase in the activated fraction can be observed. The high initial fraction of over 0.3 at time point 0 is due to the non-optimal experimental setup where C12-HSL leaked into the system before the acquisition of fluorescence images was started. For a dose response of C12-HSL/LasR controlled GFP expression in *E. coli*, please refer to Figure 5.1.

The GFP expressed from pMG401 contains a C-terminal LVA tag which makes it a target for the SsrA depended protein degradation pathway [5, 6]. GFP with this tag shows a drastic decrease in lifetime. Once the expression of GFP(LVA) is stopped, the fluorescence of *E. coli* cultures expressing it decreases (see Figure S 3.7). To confirm this degradation of GFP(LVA), we grew *E. coli* containing pMG401 in liquid culture with medium supplemented with C12-HSL. The culture was used to seed a flow channel and the flow medium lacked the C12-HSL (Figure 1 D). The signal decreases and no GFP
signal is detected after 50 minutes. This makes the pMG401 QS sensor not only suitable for detecting QS onset, but also to monitor its offset.

To determine the influence of the ribosome binding site we exchanged the strong synthetic RBSII from upstream \textit{lasR} with the native ribosomal binding sites of this gene found in the \textit{P. aeruginosa} PAO1 genome. The native RBS upstream \textit{lasR} results in a reduced sensitivity towards C12-HSL (Figure 1) which is probably due to lower expression levels of LasR. This implies, that the amount of LasR protein is crucial for the sensitivity of a synthetic QS circuit. Exchanging the ribosome binding site thus offers a possibility of fine-tuning such synthetic reporters.

Depending on their composition, bacterial growth media can exhibit considerable autofluorescence which may mask very low expression levels of GFP or the detection is delayed so that a lag time between the start of GFP expression and its actual detection builds \cite{7}. To get an idea what the extend of autofluorescence is, we acquired emission scans of different media at an excitation wavelength of GFP (unit 475 nm) compared to pure water (Figure 1 E). The data shows a considerable contribution of LB medium to green fluorescence. This should be considered when using low intensity fluorescent markers or when acquiring kinetics and drawing conclusions about when a fluorescent marker such as GFP starts to be expressed.

## 2 A Method for estimating biomass in flow channels

A common indicator of biomass in bacterial batch cultures is the turbidity measurement (known as optical density, OD if the light path length is 1 cm). This method is not applicable for flow cell experiments. To address the increase in biomass, we acquired phase contrast micrographs of surface attached bacteria in a flow chamber (Figure 2 A, top panel). The images were thresholded with the auto threshold routine of the ImageJ software (Figure 2 A, middle panel) and the fraction of pixels covered by bacteria (i.e. the thresholded pixels) was determined. This gives a good estimation of the increase in biomass for the first 150 minutes of a flow chamber experiment. As the population density increases, this method underestimates the biomass (Figure 2 A and C, green triangles). Bacterial growth in z-direction cannot be addressed at all.

We therefore limited this measurement to the first 150 minutes of the experiment. The acquired data was fitted with the equation

\[ N(t) = N_0 \cdot 2^{t/g} = e^{(\ln 2/g) \cdot t} \] (1)

where \( N_0 \) is the number of pixels covered by bacteria at time \( t = 0 \). The fit yields a generation time \( g \) (the time of one division cycle) and allowed an
2 A Method for estimating biomass in flow channels

Figure 1: (A) The quorum sensing reporter plasmid pMG401 used to transform *E. coli*. (B) Time course of C12-HSL/LasR controlled GFP expression by surface attached *E. coli* in flow chambers. 0.01 mM C12-HSL was added to the medium at time point 0 and fluorescent micrographs were acquired. (C) The fraction of bacteria expressing C12-HSL/LasR controlled GFP at different time points after addition of C12-HSL. (D) LVA dependent degradation of GFP(LVA) in *E. coli* expressing GFP(LVA) from pMG401. (E) Effect of different RBS's on the sensitivity of LasR controlled GFP towards C12-HSL. (F) Emission scan of autofluorescence of different growth media at 475 nm excitation wavelength.
extrapolation of the fit to predict a biomass at times beyond 150 minutes (Figure 2 C, red circles). This approach assumes continued bacterial growth in the logarithmic phase without entering the stationary phase. Since in a flow chamber the surface attached bacteria are continuously supplied with nutrients and metabolic waste products are transported away by the flow, this assumption was considered to be justified, at least until the flow chamber is fully overgrown.

To test this approach, we compared the predicted biomass with the fluorescence signal of GFP, which was constitutively expressed by the bacteria (Figure 2 A, bottom panel). As all bacteria are assumed to express the same amount of GFP, the fluorescence signal increases proportionally with the biomass. In widefield microscopy, the entire sample is excited and out of focus light is acquired, too. Thus, the fluorescence intensity of an image represents the entire biomass at that position, not only at the focal plane.

The sum of pixel values (integrated density) of fluorescent bacteria at each time point was measured from fluorescent images (Figure 2 C, black squares). Our results show a good fit between the predicted biomass and the biomass measured by fluorescence imaging for the first 250 minutes of the experiment. At later time points, the prediction overestimates the biomass. This can be due to increased scattering of fluorescence light through the increased biomass and by the bacteria already leaving the logarithmic growth phase.

The presented method of extrapolating the increase in surface area coverage of surface attached bacteria is suitable to estimate the increase in biomass for an extended time period. The period that can be addressed depends on the generation time of the observed bacteria and should be verified by e.g. measuring the fluorescence intensity of a constitutively expressed fluorescent protein.

3 The influence of flow velocity on gene expression

Different flow rates can affect the bacterial metabolism by providing more or less nutrients and oxygen [8]. To estimate the influence of different flow rates on *P. aeruginosa* matrix production, we grew *P. aeruginosa* in flow chambers at a flow rate of 0.6 and 1.2 μL/min and monitored matrix production in real time by addition of 20 μg/mL rhodamine labeled conA (Figure 3 A). At a flow rate of 1.2 μL/min the matrix is detected after 4 h whereas at half the flow rate the matrix is detected after 8 h.

To determine the influence of different flow rates on the induced expression of GFP, we grew *E. coli* that express GFP upon addition of *N*-butyryl-HSL (Figure 3 B). *E. coli* harboring plasmid pFNK-601 (a kind gift from Prof. Frances Arnold,[4]) were grown in a flow chamber. GFP expression was in-
3 The influence of flow velocity on gene expression

**Figure 2:** Method to estimate the bacterial biomass from phase contrast images. (A) Measuring biomass increase by the area covered by bacteria (top and middle panel) or by overall fluorescence intensity of GFP expressed by the bacteria (bottom panel). (B) The increase in area covered at low cell densities is plotted and fitted. (C) The fit from the area covered is extrapolated (red dots) and compared to the fluorescence intensity (black squares). Simply measuring the area covered underestimates the biomass increase (green triangles).
Appendix

duced by switching to a medium containing 0.01 mM \(N\)-butyryl-HSL (indicated by an arrow in Figure 3 B). At both flow rates, GFP fluorescence was detected few minutes later, however, the signal rose faster at the higher flow rate, indicating a higher metabolic rate of the bacteria. In the case of GFP (Figure 3 B), the expression was triggered externally and starts at the same time but at different kinetics, whereas in \(P.\ aeruginosa\), the matrix production was not triggered externally. There, a delay of 4 h is observed at the lower flow rate (Figure 3 A). This may be due to lower growth rates at lower flow rates since less nutrients are advected. Growth rates were not determined in this experiment.

These results suggest, that higher flow rates of medium result in a higher metabolic activity of the bacteria.

4 The influence of the \textit{lasI} gene product on \textit{psl} expression in \textit{P. aeruginosa}

The \textit{psl}-operon has been reported not to be a target of quorum sensing in \textit{P. aeruginosa} [10]. To test if the Psl EPS can be detected in \textit{P. aeruginosa} with a deletion in the \textit{lasI} gene, we grew \textit{P. aeruginosa} PAO-JP1 [9] in a flow chamber and added rhodamine labeled conA at a concentration of 20 \(\mu\)g/mL to the flow medium (Figure 3 C) and were able to detect the matrix. This suggests, that the \textit{psl}-operon is expressed independent of the LasI/R QS system. However, we did not test a \textit{rhlI} or a double mutant. Strain PAO-JP1 was kindly provided by Prof. Barbara Iglewski from the University of Rochester, NY.

5 Subcloning the \textit{fim}-cluster from pSH2 into pBR322

\textit{E. coli} can adhere specifically to mannose by their type-1-pili [11]. To study type-1-pili, a \textit{fim}-mutant was generated [12] and the \textit{fim}-cluster was subcloned into plasmid pACYC184 to provide plasmid pSH2 [13] for reconstitution. In our laboratory, much work was done on the characterization of the catch-bond nature of the type-1-pili tip adhesin FimH [14–19]. The pACYC184 vector contains a p15A replicator. However, two plasmids with the same repli-ator or repli-ators of the same incompatibility group cannot be maintained in one bacterial cell [20]. To be more flexible in the design of our experiments, we subcloned the \textit{fim}-cluster from pSH2 into pBR322. pBR322 has a MB1 replicator which is compatible with the p15A replicator.

To test the successful cloning of the \textit{fim}-cluster into pBR322, resulting in pMG201, we transformed the \textit{E. coli} \textit{fim}-mutant strain AAEC191A [12] with pMG201. A flow chamber glass bottom was coated with RNaseB, a mannose
Figure 3: (A) Influence of different flow rates on the matrix production of *P. aeruginosa* PAO1. (B) Influence of different flow rates on the induced expression of GFP under LasR control. (C) conA labeled matrix of *P. aeruginosa* PAO1 strain with a $\delta$lasI mutation (PAO-JP1, [9]) grown in a flow chamber. (D) Excitation and emission scans of eGFP and mRFP1 in pHis (see table 1). (E) Test of plasmid pMG201 containing the fim-cluster and reconstituting the $\delta$fim mutant *E. coli* AAEC191A. (F) Stamping bacteria in circular patterns on a glass surface using an agarose stamp.
presenting glycoprotein. The fim-mutant strain AAEC191A does not adhere to the surface, as it lacks the type-1-fimbria (Figure 3 E). When transformed with pMG201, the adhesion capability to RNaseB coated surfaces is reconstituted, indicating a successful incorporation of the fim-cluster into pBR322.

6 Stamping bacteria on glass surfaces

In chapter 4 we presented the photography and lift-off method (MAPL) technique to adhere bacteria to surfaces in patterns at the micron scale. To have a less laborious and faster method we were experimenting with printing bacteria directly onto a surface.

Agarose stamps were reported to stamp bacteria onto agar plates at the millimeter scale [21] and PDMS stamps are commonly used to stamp proteins onto glass surfaces at the micron scale [22]. We aimed to combine these methods and use agarose molded over SU8 photolithographic patterns at the micron scale to stamp bacteria in patterns onto a glass surface. Agarose has the advantage that a liquid put on top of it will soak fast into the gel. When pipetting a solution of bacteria onto an agarose stamp, the liquid is quickly absorbed by the stamp but the bacteria accumululate on the surface of the stamp. We used high gel strength agarose (Agarose MP, AppliChem A1091) to produce a stamp from a SU8 mold featuring circular patterns with a diameter of 100 µm. An E. coli culture (OD$_{600}$=1) was pipetted on top of the agarose stamp and incubated until no liquid was left at the surface. A RNaseB coated and dry blown surface was then inversely put on top of the stamp. The glass was removed again and observed with a phase contrast microscope (Figure 3 F). The procedure results in bacterial patterns on the glass surface. However, the area between the spots is not completely free of bacteria. Molds with thicker layers of photoresist may help to minimize the contamination of areas between the bacterial patterns. However, since these areas are not biopassivated, the procedure is not a replacement for the MAPL technique.

7 Co-cultivation of P. aeruginosa and E. coli C12-HSL receiver

We co-cultured P. aeruginosa PAO1 wild type together with an E. coli C12-HSL receiver strain that itself does not produce C12-HSL but harbors lasR from a constitutive promoter and gfp under the control of the qscrsaL quorum controlled promoter (plasmid pFNK-503-qscrsaL, kindly provided by Prof. Frances Arnold, California Institute of Technology [4]. The P. aeruginosa matrix was stained by addition of 20 µg/mL rhodamine labeled conA to the
7 Co-cultivation of *P. aeruginosa* and *E. coli* C12-HSL receiver

Table 1: All plasmids cloned during this work. Some of these were not used in experiments that were presented in chapters 3-5

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHis-GFP</td>
<td>eGFP from inducible <em>tac</em> promoter in pHis</td>
</tr>
<tr>
<td>pHis-GFP(LVA)</td>
<td>an LVA tag is added to eGFP C-terminally to decrease its lifetime [6]</td>
</tr>
<tr>
<td>pHis-RFP</td>
<td>mRFP1 from inducible <em>tac</em> promoter in pHis</td>
</tr>
<tr>
<td>pHIS-RFP(LVA)</td>
<td>an LVA tag is added to mRFP1 C-terminally to decrease its lifetime [6]</td>
</tr>
<tr>
<td>pMG101</td>
<td>pFNK-601-R with its replicator p15A replaced by MB1</td>
</tr>
<tr>
<td>pMG102</td>
<td>pFNK-602-green with its replicator p15A replaced by MB1</td>
</tr>
<tr>
<td>pMG201</td>
<td><em>fim</em>-cluster from pSH2 subcloned into pBR322</td>
</tr>
<tr>
<td>pMG402</td>
<td>contains the <em>P. aeruginosa</em> LasI/R QS system</td>
</tr>
<tr>
<td>pMG403</td>
<td>contains the <em>P. aeruginosa</em> RhlI/R QS system</td>
</tr>
<tr>
<td>pMG404</td>
<td>pMG401 with the RPSII upstream <em>lasI</em> and <em>lasR</em> replaced by the corresponding native RBS found in the <em>P. aeruginosa</em> PAO1 genome</td>
</tr>
<tr>
<td>pMG405</td>
<td>pMG403 C1418T</td>
</tr>
<tr>
<td>pMG406</td>
<td>pMG404 C747T</td>
</tr>
<tr>
<td>pFNK-503-nRBS</td>
<td>pFNK-501-qacsraL [4] with the RBSII upstream <em>lasR</em> replaced with the native RBS of <em>lasR</em> found in the <em>P. aeruginosa</em> PAO1 genome</td>
</tr>
</tbody>
</table>

flow medium (Figure 4 A). The *P. aeruginosa* does not express any fluorescent marker and is not visualized. *E. coli* expresses GFP when the threshold concentration of C12-HSL, which is produced by *P. aeruginosa*, is reached. Interestingly, the subtly larger *E. coli* is not incorporated in the *P. aeruginosa* but rather sits on top of it or in between layers of matrix (Figure 4 B). The onset of quorum sensing controlled GFP expression in the *E. coli* receiver is strongly dependent on the surrounding medium flow. Only at static conditions GFP signal is observed. Under flow conditions, not sufficient C12-HSL can accumulate locally to induce GFP expression in the *E. coli* since it is not incorporated into the matrix (see chapter 5).
Figure 4: Maximum intensity projections (MIP) of confocal microscopy Z-stacks of *P. aeruginosa* PAO1 wild type (not labeled) in its matrix (red) and *E. coli* C12-HSL receiver (green). (A) x-y and corresponding x-z and y-z MIP. (B) x-z reslices (MIP) of 2 different stage positions.
8 Vector map of pMG401

Annotation:

67-786 gene lasR (complementary)
796-897 promoter laqI-q
907-936 promoter qscraL
985-993 RBSII
1003-1608 gene lasI
1713-1721 RBSII
1731-2486 gene gfp(lva)
2531-2635 terminator T1
2789-3351 replicator p15A
3445-3550 terminator T0
3575-4369 gene kan (complementary)

pMG401

1 ccaaccttaccagagggcgccccagctggcaattccgacgtcattgcgtgcacctgagaggcaagatcag

8 Vector map of pMG401
Appendix

ctcgccccccctgacacagcatcagcacaatctgcagctcaatcagtggtggcgaaacccgacaggactat
aaagataccaggcgtttccccctggcggctccctcgtgcgctctcctgttcctgcctttcggtttaccgg
tgtcattccgctgttatggccgcgtttgtctcattccacgcctgacactcagttccgggtaggcagttcg
tccaagctggactgtatgcacgaaccccccgttcagtccgaccgctgcgccttatccggtaactatcgt
cggagttcccccggcgttcgctccaaatcggcagccggctcctgcagttcattca
ctggggaagcgggttactagtgctttggattctcaccaataaaaaacgcccggcggccagcgttctgaacaaatc
cagatggagttctgaggtcattactggatctatcaacaggagtccaagcgagctctcgaaccccagagtc
cctgatagcggtccgccacacccagccggccacagtcgatgaatccagaaaagcggccattttccacca
gccatattccggcaagcagggcatcgcctgcctgcagttcattca
cggcaccggacaggtcgggtcttgacaaaaagaaccgggcgcccctgcgctgacagccggaacacggcggc
cctgcgtgcaatccatcttgttcaatcatgcgaaacgatcctcatcctgtctcttgatcagatcttgatc
cctgcgccatcagatccttggcggcaagaaagccatccagtttactttgcagggcttc
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construction of new fim deletion mutants”. In: *Molecular Microbiology* 5.6 (1991), pp. 1439–1445.


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Publication list

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Philippe Emge, Jens Möller, Hongchul Jang, Roberto Rusconi, Yutaka Yawata, Roman Stocker and Viola Vogel. “Biofilm matrix rescues bacterial quorum sensing under flow” submitted to PNAS