**E. coli** biofilm formation and its susceptibility towards T4 bacteriophages studied in a continuously operating mixing – tubular bioreactor system

Ana Lisac1, Elfı Birsa1 and Aleš Podgornik1,2*

1Faculty of Chemistry and Chemical Technology, University of Ljubljana, Večna pot, 113, Ljubljana, Slovenia.
2COBIK, Mirce 21, 5270, Ajdovščina, Slovenia

Summary

A system consisting of a connected mixed and tubular bioreactor was designed to study bacterial biofilm formation and the effect of its exposure to bacteriophages under different experimental conditions. The bacterial biofilm inside silicone tubular bioreactor was formed during the continuous pumping of bacterial cells at a constant physiological state for 2 h and subsequent washing with a buffer for 24 h. Monitoring bacterial and bacteriophage concentration along the tubular bioreactor was performed via a piercing method. The presence of biofilm and planktonic cells was demonstrated by combining the piercing method, measurement of planktonic cell concentration at the tubular bioreactor outlet, and optical microscopy. The planktonic cell formation rate was found to be $8.95 \times 10^{-3} \text{ h}^{-1}$ and increased approximately four-fold ($4 \times$) after biofilm exposure to an LB medium. Exposure of bacterial biofilm to bacteriophages in the LB medium resulted in a rapid decrease of biofilm and planktonic cell concentration, to below the detection limit within $< 2$ h. When bacteriophages were supplied in the buffer, only a moderate decrease in the concentration of both bacterial cell types was observed. After biofilm washing with buffer to remove unadsorbed bacteriophages, its exposure to the LB medium (without bacteriophages) resulted in a rapid decrease in bacterial concentration: again below the detection limit in $< 2$ h.

Introduction

Bacterial biofilms are dynamic structures of bacteria (Costerton et al., 1999; Azeredo et al., 2021) that develop on inert surfaces, such as medical implants and medical devices (Donlan, 2001, 2002), dead and living tissues (Lambe et al., 1991; Costerton et al., 1999), and pose a serious global threat due to their tolerance to host immune defences and antibiotic treatments (Marrie et al., 1982; Costerton et al., 1999; Hall-Stoodley et al., 2004; De la Fuente-Núñez et al., 2013; Sharma et al., 2019). These structures are clusters formed by single (Tolker-Nielsen et al., 2000; Su et al., 2012) or multiple bacterial species (Burmølle et al., 2006) that are generally less metabolically active and therefore less affected by antibiotics (Davies, 2003; Stewart, 2015; Sønderholm et al., 2017). Bacteria are connected within a biofilm matrix consisting of extracellular polymeric substances (EPS), such as exopolysaccharides, extracellular DNA, and proteins (Zhang et al., 1998; Sutherland et al., 2004; Flemming et al., 2007; Flemming and Winfield, 2010), which are produced by the bacteria in response to the absence of a substrate in the environment and serve as a physical barrier.

Biofilms play a significant role in persistent inflammation (Costerton et al., 2003; Jamal et al., 2018; Svensson Malchau et al., 2021) and antibiotic tolerance, (Sharma et al., 2019) as they are formed to protect bacteria and grant survival of a community in an inhospitable environment. Biofilm formation begins with the adsorption of free-floating bacteria to a surface and continues with bacterial adhesion (Davies et al., 1993; Davies and Geesey, 1995; Tuson and Weibel, 2014). Due to adverse conditions and stress signals, biofilm development must be rapid to prevent the death of the bacterial population. The expression of genes to stimulate the biosynthesis of adhesion and EPS components occurs; (Whiteley et al., 2001; Beloin and Ghigo, 2005) once strong adhesion is attained, bacteria begin to grow, divide, accumulate, and form an EPS matrix for structural support and protection (Keleștemur et al., 2018).
The composition of the EPS matrix varies from biofilm to biofilm but typically accounts for approximately 50–90% of the biofilm (Flemming and Wingender, 2010; Dhayakaran and Neethirajan, 2017; Jamal et al., 2018). With EPS production and bacterial growth, the biofilm begins to mature and thicken, generating multiple layer structures that differ between environmental conditions and bacterial species (O'Toole and Kolter, 1998; Vu et al., 2009). Once a biofilm has developed, the detachment of biofilm parts due to shear or mechanical stress, or bacteria-regulated multiplication and dispersion of planktonic bacteria occurs to continue the colonization of new surfaces (Stoodley et al., 2001; Sauer et al., 2002; Hall-Stoodley et al., 2004; De la Fuente-Núñez et al., 2013).

Mature biofilms cause a range of chronic infections, since their eradication is extremely difficult (Lewis, 2001). Biofilms are also a source of planktonic bacteria that can cause acute infections and are susceptible to antibiotic therapy. New planktonic bacteria can be released from biofilms constantly, causing a recurrence of symptoms (Read et al., 1989; Costerton et al., 1999, 2003).

To avoid several antibiotic treatments and the possibility of antibiotic resistance, new, efficient anti-biofilm therapies are needed. Bacteriophages (‘phages’ for short) represent suitable anti-biofilm agents (Doolittle et al., 1995; Alves et al., 2016; Forti et al., 2018; Hansen et al., 2019). Lytic phages are viruses that use bacteria for their propagation and kill them (Ackermann and Prangishvili, 2012). In terms of biofilm removal, phages show interesting properties as they produce enzymes that facilitate their penetration into bacterial biofilm and eventually its disruption (Hughes et al., 1998a,b; Abedon, 2015; Simmons et al., 2018). Besides, they are specific, allow automatic dosing at the infection site, and avoid the occurrence of side effects when used for therapeutic purposes. However, in order to use phages as therapeutic anti-biofilm agents, it is necessary to understand their adsorption, infection, propagation, and lysis, to optimally exploit their potential for biofilm eradication. This is of significant importance since the efficacy of phages against bacterial biofilms depends on the biofilm structure (Sutherland et al., 2004).

To investigate phage efficacy against bacterial biofilms under controlled conditions, a continuously operating system consisting of a mixing bioreactor, operating like a chemostat, and a tubular bioreactor, connected in series was developed. Proof of principle was demonstrated using phage T4 and bacteria host Escherichia coli as a model system. The bacterial biofilm was formed on the inner wall of the tubular silicone bioreactor under defined conditions, at a constant temperature, linear velocity, and bacterial physiological state during supply, determined by chemostat dilution rate. This set-up enabled studying the properties of the bacterial biofilm and its eradication when exposed to phages in the presence of substrate or under substrate limitation.

Results and discussion

Experimental set-up

The experimental set-up shown in Fig. 1A was developed to investigate the formation of E. coli bacteria biofilm and its eradication using phages. It consisted of a chemostat, providing the flow of bacteria in a constant physiological state, SM buffer flow, and a silicone tubular bioreactor mimicking in particular case silicone urinary catheter (Read et al., 1989; Stickler, 1996; Donlan, 2001; Koseoglu et al., 2006; Wilks et al., 2021) inside which a biofilm can be formed. The sampling of bacteria along the tubular bioreactor was performed by the piercing method, which consisted of several steps (Fig. 1B). First, a thin, sterile syringe needle was used to pierce the bioreactor wall at various positions. Due to the small needle diameter (OD 0.35 mm), the elastic silicone tubing spontaneously sealed after the needle was removed, preventing contamination. When the needle penetrated the silicone tubing entering into a tubular bioreactor (Fig. 1B), it was assumed that a small portion of the biofilm was cut and remained in the needle. Once the needle was inside the bioreactor, an additional 50 μl of the medium was collected, potentially containing free-floating bacteria. In this way, both types of bacteria could be sampled simultaneously together with unadsorbed phages (when present). Set-up enabled studying biofilm formation and the effect of its exposure to phages, according to the design of the experiments presented in Fig. 1C.

Biofilm formation

To form a bacterial biofilm, E. coli bacteria in LB medium from the chemostat were continuously mixed with SM buffer (1:1 ratio – Fig. 1A) and allowed to adhere to the inner surfaces of the tubular bioreactor. With the LB medium, an essential concentration of nutrients for the formation and growth of bacterial clusters on the walls was provided, initiating biofilm growth and EPS production (Guzmán-Soto et al., 2021), while the SM buffer was used to enhance growth-limiting conditions. Bacteria were supplied into the tubular bioreactor for 2 h at a flow rate of 0.2 ml min⁻¹, corresponding to a linear velocity of 0.01 m min⁻¹, providing laminar flow conditions.

During the bacterial supply, samples were taken from different positions of the tubular bioreactor by the piercing method, representing different residence times τ(θ) of the bacteria inside the bioreactor. The bacterial...
concentration determined 1 h after the start of continuous bacteria supply, shown in Fig. S1, demonstrates that almost constant bacterial concentration along the tubular bioreactor was present. This indicated that cell adsorption on the inner wall was slow (if present at all), as no constant decrease in concentration along the bioreactor was observed. Constant bacterial concentration is expected to result in the formation of a uniform bacterial biofilm throughout the tubular bioreactor.

After 2 h of continuous bacteria supply in diluted medium, the bacterial flow was stopped and replaced by solely SM buffer flow to remove unadsorbed bacteria, limit nutrient supply and therefore promoting biofilm formation on the inner wall of the tubular bioreactor (Flemming et al., 2007; Sharma et al., 2019). Washing with SM buffer continued for 24 h, allowing biofilm maturation. Bacterial biofilms also generate planktonic bacteria (Ward et al., 1992; Costerton et al., 1999, 2003; Mah and O'Toole, 2001; Hall-Stoodley et al., 2004; Bjarnsholt, 2013) that spread potential bacterial infections (Sharma et al., 2016) and cause acute infections (Bjarnsholt, 2013). Because of that, it is important to monitor both, concentration of biofilm and planktonic bacteria. Total bacterial concentration during washing was monitored by the piercing method at various residence times along the bioreactor (Fig. 2A).

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The data, based on the analysis of three independent experiments, showed that the bacterial concentration in the tubular bioreactor decreased until a steady state was reached somewhere between the 3 and 22 h. As no new bacteria were introduced into the tubular bioreactor during washing, detected bacteria should represent potential biofilm (XB) and/or planktonic (XP) bacteria. To discriminate between the two types, the bacterial concentration at the bioreactor outlet (XP outlet) was determined by collecting the outlet medium after 24 h of washing, containing therefore only free-floating planktonic bacteria. Their concentration was determined to be $2.63 \times 10^6$ CFU ml$^{-1}$. Subtracting this value from the total bacterial concentration determined by the piercing method at the bioreactor end ($\tau = 0.75$ h) resulted in a concentration of $9.97 \times 10^5$ CFU ml$^{-1}$, representing biofilm bacteria (XB). Interestingly, this value is similar to the total bacterial concentration obtained by the piercing method at the beginning of the bioreactor ($\tau = 0$ h), namely $(0.73 \pm 0.28) \times 10^6$ CFU ml$^{-1}$. Since no free bacterial cells enter the tubular bioreactor, the total bacteria concentration at $\tau = 0$ h should therefore represent concentration of biofilm bacteria (XB$_0$). The similar concentration of biofilm bacteria at the beginning and end of
the tubular bioreactor confirmed that a uniform biofilm has formed throughout the tubular bioreactor, as could be anticipated from a constant bacteria concentration along the bioreactor during their supply (Fig. S1). This conclusion is also supported by microscopic images of biofilm from different positions (Fig. S2). Uniform biofilm explains the linear increase in total bacteria concentration along the tubular bioreactor (Fig. 2B). If planktonic cells are formed by bacteria biofilm uniformly, their generation throughout the bioreactor is constant what results in the observed trend.

To investigate whether the bacteria detected at the outlet of the tubular bioreactor were planktonic bacteria or detached biofilm aggregates, the bacterial sample was examined with an optical microscope. Only single floating cells without aggregates were observed (data not shown). This could be anticipated due to a constant, laminar flow regime within the bioreactor (Reynolds number = 0.51) causing a shear rate of only 17.06 s⁻¹ and shear stress of only 0.026 Pa on the bacterial biofilm; therefore, no biofilm disruption is expected. To investigate the morphology of the biofilm on the inner silicone wall, optical microscopy was again applied (Fig. 3 A and B).

The images show the biofilm presence on the inner part of the silicone wall that was of non-uniform thickness. Nevertheless, to simplify its description, the average thickness was determined from the images and found to be around 13 μm. Doing so enabled the estimation of cell packing within the biofilm. E.coli bacteria are rod-shaped (Reshes et al., 2008) with an approximate size of 1 × 1 × 2 μm and a volume of 2 μm³ (Riley, 1999). Bacteria were sampled from an area corresponding to the cross-section of the inlet needle (Pneedle = 7.06858 × 10⁻⁸ m²), and the average content of sampled bacteria was 3.45 × 10⁴ CFU (6.91 × 10⁵ CFU ml⁻¹ × 50 μl). In the case of tight bacteria packing within the biofilm, 7.07 × 10⁻² mm² of the biofilm with an estimated thickness of 13 μm would contain 4.59 × 10⁵ bacteria. The ratio between the measured bacteria in the biofilm and maximal bacteria number at tight packing represents the volumetric fraction occupied by bacterial cells in the actual biofilm. The obtained value of 7.52% fits within the range of values observed in bacterial biofilms (Corbin et al., 2001; McLean et al., 2001; Flemming and Wingender, 2010).

Based on the demonstrated presence of the biofilm, it seems reasonable to conclude that the planktonic cells are generated by biofilm bacteria. Therefore, it would be worthwhile to estimate their dispersion rate. This parameter can be calculated by dividing the amount of planktonic bacteria exiting the tubular bioreactor by the amount of biofilm bacteria.

The total amount of biofilm bacteria can be estimated from the amount of bacteria sampled by the piercing method at the τ = 0 h of the tubular bioreactor (3.45 × 10⁴ CFU on the area of Pneedle = 7.06858 × 10⁻⁸ m²), extrapolating it to the total inner wall area. Considering that uniform biofilm is present and the total inner surface area of the tubular bioreactor (Fig. 1B) is 7.226 × 10⁻³ m², one can calculate the total amount of biofilm bacteria in the bioreactor to be 3.53 × 10⁸. Since the concentration of planktonic bacteria exiting the tubular bioreactor was 2.63 × 10⁶ CFU ml⁻¹ (Fig. 2C) at a flow rate of 0.2 ml min⁻¹, approximately 5.27 × 10⁵ planktonic cells have been generated every minute (3.16 × 10⁷ CFU h⁻¹). The ratio between the planktonic bacteria formation rate and total biofilm bacteria provides an average planktonic cells generation rate of

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![Fig. 3. Image of bacterial biofilm after 24 h of continuous SM buffer flow through the tubular bioreactor on optical microscope (A) under transmitted and (B) under reflected light.](image-url)
8.95 \times 10^{-3} \text{ h}^{-1}. On the other hand, constant bacterial biofilm concentration between 22 and 24 h of SM buffer washing (Fig. 2A) indicated that all bacterial cells formed by the biofilm were released into the medium as planktonic bacteria.

**Effect of LB medium on formed biofilm**

To investigate the viability of formed bacterial biofilm, the LB medium flow was introduced into a tubular bioreactor, and the bacterial concentration was monitored by the piercing method (Fig. 4).

A significant increase in bacterial concentration along the bioreactor can be observed after substrate addition, indicating faster generation of planktonic bacterial cells. In addition, the higher bacterial concentration at the entrance of the bioreactor ($\tau = 0$ h) also indicated an increase in bacterial biofilm concentration after the addition of fresh nutrients (Fig. 4).

To estimate the generation rate of bacteria inside the tubular bioreactor after substrate addition, we can presume that the bacterial concentration at $\tau = 0$ h represents the biofilm bacteria ($1.366 \times 10^7$ CFU ml$^{-1}$). Therefore, the concentration of planktonic bacteria along the bioreactor can be calculated by subtracting the biofilm cells from the total bacteria concentration at $\tau = 0.75$ h ($1.91 \times 10^8$ CFU ml$^{-1}$), again assuming that the biofilm is uniform along the bioreactor. The number of biofilm bacteria inside the tubular bioreactor after LB addition is $6.98 \times 10^{10}$. Since $3.82 \times 10^7$ planktonic bacteria were generated every minute, their formation rate increased to $0.0328$ h$^{-1}$ after LB addition, which is almost 4-fold higher compared to the substrate limitation conditions.

**Phage interaction with bacterial biofilm**

**Application of phages in LB medium.** Phages, as bacterial viruses, have interesting properties in terms of biofilm control and elimination, as they can invade and affect the structure of biofilms (Abedon, 2011), multiply at the site of infection, and lyse bacteria. Phage multiplication is greatly affected by the physiological state of planktonic bacteria (Hadas et al., 1994, 1997; Abedon et al., 2001; Golec et al., 2014; Nabergoj et al., 2018b). Phages replicate more effectively in bacterial cells cultivated under optimal growth conditions, whereas they have little or no effect in bacteria with reduced metabolism and low specific growth rate (Abedon et al., 2001; Łoś and Wegrzyń, 2012; Cenens et al., 2013). One can speculate that this phenomenon would play an important role also for biofilms where bacteria of different physiological state are present (Stemberg et al., 1999) and affected by nutrients (Stoodley et al., 1999).

To investigate phage adsorption, infection of the biofilm and phage propagation, the formed biofilm was exposed to phages by pumping them through the tubular bioreactor in LB medium, which provided the necessary nutrients for the biofilm bacteria. Phage and bacteria concentrations were monitored along the bioreactor by the piercing method for several hours after the start of LB medium flow containing phages (Fig. 5).

Experimental data showed that the initial concentration of unadsorbed phages in LB medium 1 h after the start
of their supply was substantially lower than their loading concentration, indicating intense adsorption to the bacterial biofilm (Fig. 5A). In contrast, the increase in phage concentration along the bioreactor indicated their multiplication, demonstrating bacterial infection and lysis. This is further confirmed by the phage concentration above the loading concentration after the 2 and 3 h. Significantly, no uninfected bacteria were detected in the tubular bioreactor after only 2 h of phage supply (Fig. 5B), demonstrating their complete infection. Phages supplied together with LB medium were, therefore, able to eradicate the bacterial biofilm in the tubular bioreactor, showing plausible phage efficacy on biofilms in the presence of bacterial nutrients.

**Application of phages in SM buffer**

Bacterial biofilms are usually present in sub-optimal environments for bacterial growth (Ward et al., 1992), which might result in a substantially decreased phage multiplication or even its absence in planktonic cells, due to their low-metabolic activity (Sutherland et al., 2004; Golec et al., 2011; Nabergoj et al., 2018b). To examine phage effect under such conditions on biofilm, phages suspended in SM buffer were introduced, providing no bacterial nutrients. Unadsorbed phage concentration at the tubular bioreactor outlet was monitored over time (Fig. 6).

Since loading phage concentration was $9.00 \times 10^6$ PFU ml$^{-1}$, it can be concluded that the phages were initially adsorbed to the biofilm, while approximately 1.85 h after the start of loading (1.1 h if residence time of 0.75 h within bioreactor is considered) phage concentration similar to inlet one was reached at bioreactor outlet. Although lower initial phage concentration indicated phage adsorption, there is no clear evidence of their multiplication. Considering the difference in the average phage concentration at bioreactor outlet and inlet, one can calculate the amount of adsorbed phages according to the following equation:

$$N_P = t \cdot F (ml \ min^{-1}) \times P_0 \times \left( \frac{P_{\text{aver}}}{P_0} \right)$$

$$= (1.85 - 0.75) \times 60 \ min \ h^{-1} \times 0.2 \ ml \ min^{-1} \times$$

$$9.0 \cdot 10^6 \text{PFU ml}^{-1} \times 0.9 \times$$

$$9.0 \cdot 10^6 \text{PFU ml}^{-1} \times 0.9 = 1.07 \cdot 10^8 \text{PFU}$$  \hspace{1cm} (1)

From the number of adsorbed phages ($1.07 \times 10^8$) and total concentration of bacteria, both biofilm and planktonic, MOI (multiplicity of infection) can be estimated (Equation 2):

$$\text{MOI} = \frac{P}{X} = \frac{1.07 \cdot 10^8 \text{PFU}}{3.54 \cdot 10^9 \text{CFU}} = 0.0302$$  \hspace{1cm} (2)

This calculation assumed that all biofilm bacteria are equally accessible to phages, which is not a realistic assumption since biofilm thickness was found to be in average of 13 μm and therefore biofilm bacteria are present in several layers. More realistically, only the upper bacterial layer is initially accessed. Considering biofilm thickness and bacteria dimensions, the upper biofilm layer consisted of approximately $2.72 \times 10^8$ CFU, resulting in an estimated MOI of 0.39. Real MOI value is

![Fig. 5. Concentration of unadsorbed phages and uninfected bacteria during several hours of continuous phage supply in LB medium.](image-url)
probably between these two values what demonstrates that only small portion of biofilm bacteria are initially infected. Therefore, it is informative to monitor the concentration of uninfected bacterial cells along the bioreactor over several hours of phage loading in SM buffer (Fig. 7A).

Compared to a decrease in bacterial biofilm concentration, when phages were supplied in LB medium, the increase in infection was much slower in this case, as uninfected bacterial cells were still present after 3 h of phage supply. Interestingly, the initial concentration of 7.25 × 10^5 CFU ml⁻¹ dropped to 2 × 10^4 CFU ml⁻¹ (below 3%) over this period, indicating the progressive infection of the biofilm through the layers. This might be due to phage diffusion through EPS or slow upper bacterial layer lysis, enabling phage penetration deeper into the biofilm. Discrimination between the two mechanisms is not possible solely from the measurement of phage concentration at the bioreactor outlet due to a slow dynamics of this process and consequently small concentration differences. To investigate the mechanism of infection progression, the phage supply was stopped, and the infected biofilm was washed with SM buffer for 22 h to elute all unadsorbed phages. The concentration of unadsorbed phages and uninfected bacteria after 22 h of washing along the tubular bioreactor was determined by the piercing method (Fig. 7B).

The data in Fig. 7B show the presence of uninfected bacterial cells even after 22 h of SM washing. Again, to discriminate between uninfected planktonic bacteria and uninfected biofilm bacteria, the concentration of uninfected planktonic bacteria (3.90 × 10^5 CFU ml⁻¹) and infected planktonic bacteria (2.50 × 10^5 PFU ml⁻¹) were determined from the outlet medium. The presence of both types of planktonic bacteria indicated that biofilm was still present and active. Evidently, it was not eliminated as in the case of the LB medium. Nevertheless, the increase of free phage concentration along the bioreactor (Fig. 7B) and their presence at the bioreactor outlet (determined to be 1.60 × 10^6 PFU ml⁻¹) indicated their weak multiplication, which can most probably be attributed to the slow lysis of the bacterial cells.

The presence of uninfected bacteria (Fig. 7B) demonstrated low phage efficiency under such conditions. As previously calculated, the bacteria in the biofilm under substrate limitation exhibited extremely slow growth of 8.95 × 10⁻³ h⁻¹; hence, bacteria may not possess sufficient energy for phages to propagate efficiently. Under such conditions, phage propagation in planktonic cells is significantly reduced (Golec et al., 2014; Nabergoj et al., 2018b), or even causes phage hibernation, in which phages infect bacteria in the stationary growth phase, inject the phage genome and initiate protein synthesis, however further phage development is stopped until new energy for bacteria is provided (Bryan et al., 2016). Such impaired phage activity has also been reported for phages other than T4 infecting *Pseudomonas fluorescens* (Sillankorva et al., 2004) and *Staphylococcus epidermidis* (Cerca et al., 2007). Interestingly, it was recently discovered that after prolonged exposure of a stationary culture of *Staphylococcus epidermidis* to bacteriophage SEP1, bacterial cells were efficiently lysed despite reduced phage multiplication (Melo et al., 2018). This work reveals the complexity...
of interactions between bacteria and phages and shows that experiments should be performed on a particular system.

Therefore, further experiments with phage-infected and washed biofilm were conducted to investigate whether phages that may be present in the biofilm can be effective when more favourable conditions for bacterial growth occur. A constant flow of LB medium without phages was supplied to the tubular bioreactor containing the infected biofilm and, since bacterial biofilm susceptibility to fresh nutrients has already been demonstrated by an increased specific growth rate (Fig. 4), the addition of the LB medium might also result in significantly faster phage propagation. Phage and bacterial concentrations along the bioreactor and at the bioreactor outlet were monitored after the fresh substrate supply (Fig. 8).

By monitoring the concentration of uninfected bacteria along the tubular bioreactor after the start of the continuous LB medium supply, one can see that the number of bacteria was rapidly decreasing over time (Fig. 8A) to undetectable levels after only 2 h. An increase in phage concentration over time can be seen, both along the bioreactor (Fig. 8B) and at the bioreactor outlet (Fig. 8C), confirming bacterial lysis and the release of new unadsorbed phages capable of further infection to finally eradicate bacterial biofilm. This demonstrated that phage propagation is deeply connected to bacterial physiological state and confirmed potential of phages that remained inside the biofilm (either hibernating or just physically entrapped) to lyse both planktonic and biofilm bacteria when an energy source is provided.

The development of an experimental set-up consisting of connected continuously operating mixing and tubular bioreactors enabled the reproducible formation of a bacterial biofilm and, in combination with the piercing method, the monitoring of bacterial and phage profiles along the tubular bioreactor. Proof of principle was demonstrated on model microorganisms, since a stable Escherichia coli biofilm was formed within 24 h after bacterial supply and subsequent buffer washing. Results confirmed that phage efficacy is highly dependent on the physiological state of the biofilm bacteria, and reduced phage multiplication was observed under bacteria starvation conditions, similar as for planktonic bacteria. Due to its simplicity and versatility, the presented experimental set-up can be used as a platform approach to study bacterial biofilm formation, generation of planktonic cells and the effect of phage treatment, under different growth conditions.

**Experimental procedures**

**Bacteria, phage, and media**

The bacteria strain Escherichia coli K-12 MG1655 (DSM 18039) from the Leibniz Institute DSMZ Germany was used for biofilm formation and titration of the T4 lytic phage (DSM 4505). Bacteria for phage titer determination and initial inoculation of the chemostat were prepared in laboratory flasks in Lysogeny Broth (LB) (LLG Labware, Meckenheim, Germany) and incubated overnight at 37°C (Sambrook and Russel, 2001). An LB medium was used to grow the continuous bacterial culture in the chemostat and biofilm formation. For biofilm...
washing and determination of phage titer, an SM buffer (1 g gelatin (Sigma-Aldrich, St. Louis, MO, USA), 5.8 g NaCl (Merck KGaA, Darmstadt, Germany), 2 g MgSO₄ ⋅ 7H₂O (Merck KGaA, Darmstadt, Germany), 50 ml 1 M Tris–HCl (pH 7.5) (Fisher Scientific F, Loughborough, UK), deionized water to 1 l) was used.

**Bacteria and phage enumeration**

Enumeration of *E. coli* bacteria was done using an agar layer method, and the number of bacteria was expressed as colony-forming units (CFU), while phage enumeration was done using the standard double agar overlay plaque assay, and their concentration was determined as plaque-forming units (PFU) (Clokie and Kropinski, 2009) by filtering the sample through a sterile 0.22 μm syringe filter (LLG Labware, Meckenheim, Germany). All assays were performed in triplicate and average value was calculated.

**Continuous cultivation of free-floating bacteria**

*Escherichia coli* bacteria were cultivated at 37°C in a chemostat system consisting of a glass bioreactor with 25 ml working volume stirred with a magnetic stirrer at 350 rpm, as described by Nabergoj et al. (2018a). The LB medium saturated with prefiltered air was continuously supplied to the growing bacteria in the bioreactor at a flow rate of...
0.1 ml min\(^{-1}\), corresponding to a dilution rate of 0.24 h\(^{-1}\). The concentration of bacteria was monitored by online measurement of optical density at a wavelength of 600 nm. After 10 bacterial generations of continuous growth, a steady state was reached, and the concentration of bacteria in the bioreactor was determined.

Biofilm experiments

To study the interaction of bacterial biofilms with phages, a series of experiments were performed (Fig. 1C) using continuously operating systems at 37°C (Fig. 1A). A constant flow rate of 0.2 ml min\(^{-1}\) through a tubular bioreactor (a silicone tube of 0.46 m length and 5 mm inner diameter) enabled a stable flow regime in the bioreactor, while changes in the composition of the inlet flow enabled studying the effect of different conditions. The residence time within the tubular bioreactor was 0.75 h.

Biofilm formation. The LB medium from the chemostat containing approximately \(9.0 \times 10^7\) bacterial cells per mL was continuously combined with a stream of SM buffer and fed into the tubular bioreactor for 2 h to allow bacterial cell adhesion. This was followed by extensive washing with the SM buffer for 24 h to remove unadsorbed bacterial cells and to reduce the metabolic activity of the attached cells due to a substrate limitation. This procedure resulted in the formation of a reproducible biofilm that was used for all further experiments. Three independent experiments were performed in duplicate and average value and standard deviation were calculated.

The bacterial cell concentration inside the tubular bioreactor during biofilm formation was monitored via a piercing method, sampling the volume of 50 μl with the use of sterile syringe needles (Chirana T. injecta) of 1 ml working volume (see Results and discussion section for a detailed description of the method) after 1 h of bacteria supply and after 1, 2, 3, 22, and 24 h of SM buffer wash on different positions along tubular bioreactor. The concentration of only planktonic bacteria was determined in the medium exiting the tubular bioreactor.

Application of LB medium to formed biofilm. To assess the viability of the formed biofilm, only the LB medium was introduced into the tubular bioreactor. Bacterial concentration was monitored along the tubular bioreactor after 2 h of continuous LB medium supply using the piercing method. Two independent experiments were performed in duplicate and an average value was calculated.

Application of phages in LB medium to formed biofilm. Phage concentration of \(9 \times 10^6\) PFU ml\(^{-1}\) in SM buffer was continuously supplied to the biofilm. After 1, 2, and 3 h of phage supply, the concentration of unadsorbed phages and uninfected bacteria inside the tubular bioreactor was determined by the piercing method. Unadsorbed phages were determined by filtering the sample through a sterile 0.22 μm syringe filter. Two independent experiments were performed in duplicate and average value was calculated.

Application of phages in SM buffer to the formed biofilm. Phage concentration of \(9 \times 10^6\) PFU ml\(^{-1}\) in SM buffer was continuously supplied to the biofilm for 4 h. Due to specificity of experimental set-up, detailed discussion about MOI estimation is presented in section Application of phages in SM buffer. After 1, 2, and 3 h of phage supply, the concentration of unadsorbed phages and uninfected bacteria inside the tubular bioreactor was determined by the piercing method. Samples were also collected at the tubular bioreactor outlet to determine uninfected and infected planktonic bacteria and unadsorbed phages, with infected planktonic bacteria determined by a double agar overlay plaque assay without filtering the sample.

The bacterial biofilm infected by phages in the SM buffer was subsequently washed with the SM buffer without phages for 16 h to remove unadsorbed phages. The concentrations of uninfected planktonic bacteria, infected planktonic bacteria, and unadsorbed phages were determined from the bioreactor outlet medium after washing was completed. After that, the SM buffer was replaced with LB medium to provide nutrients to the bacteria in the bioreactor. The concentration of uninfected planktonic bacteria and unadsorbed phages was monitored every 15 min for 4 h in the bioreactor outlet medium. The concentration of unadsorbed phages and uninfected bacteria along the tubular bioreactor was monitored after 1, 2, and 3 h by the piercing method. Three independent experiments were performed in duplicate and average value was calculated.

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Conflict of interest

There are no conflicts of interest with the authors of this work.
Authors contributions

AL and AP contributed to the design of the research. AL and EB carried out the experiments and measurements. AL and AP analysed the results and prepared the manuscript.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Concentration of E. coli in the tubular bioreactor after 1 h of continuous bacterial feeding. A stream consisting of SM buffer and bacteria in LB medium was supplied at 0.2 ml min\(^{-1}\). Bacteria were sampled by the piercing method at different positions of the tubular bioreactor, corresponding to residence times (\(\tau\)) from \(\tau = 0\) h (at the beginning of the bioreactor) to \(\tau = 0.75\) h (end of the bioreactor). Bacterial concentration is presented with error bars as CFU ml\(^{-1}\).

Fig. S2. Microscopic images of bacterial biofilm after 24 h of continuous SM buffer flow through the tubular bioreactor under transmission light at residence times (A) 15 min, (B) 30 min and (C) 45 min.

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