Destroying Escherichia coli grown in biofilms using lysogenic bacteria

F.Bernardon*, M. Borry*, A. El Assimi*, M.Pothier*, A.Safsaf*

*all authors contributed equally to this work.
Corresponding author: maxime.borry@gmail.com

Abstract
Biofilms are a major healthcare issue and it is difficult to get rid of them considering their structure. One way to destroy them is to use lytic phages which are bacterial viruses.

We designed an alternative way of destroying an E.coli biofilm by using lysogenic bacteria (lysogens) of the phage lambda and did a mathematical model to study the bacterial population dynamics of this method.

Our experimental results show that inducing lysogens kills wild-type bacteria, even though less efficiently than with lytic phages. The model indicates that the more we wait to induce, the less living bacteria remain, and the faster we reach this minimum. Moreover, the more phages are released per bacterium, the smaller is the minimum percentage of living, and the shorter is the destruction time.

Keywords: Phages, Biofilm, Lysogens, Model, E.coli, Lambda

INTRODUCTION
Bacterial biofilms are found in nature1, but also in artificial environments. They consist of a bacterial network embedded in an ExoPoly-Saccharide (EPS) matrix2. This living form allows a greater communication between cells, thus leading to an increased physical and antibiotic resistance3.

However, because they can carry pathogenic bacteria, some biofilms are dangerous to humans. This is a problem, especially in medical environment.

Moreover, multi-resistant bacteria such as E.coli O104:H4 or Pseudomonas, responsible for cystic fibrosis, came back to the foreground recently, leaving very few solutions to fight them4.

This is why other destruction methods than antibiotics must be developed.

One solution is to use bacterial viruses, called phages5. They are numerously found in nature, in two states:

- Virulent state. They replicate inside the host in the so called lytic cycle, thus killing the bacteria.
- Temperate state. They incorporate their DNA into the bacterial genome and remain in a dormant state. This kind of bacterium is called a lysogen.

Many studies have been carried out on the interaction between virulent phage and biofilms since 1995 with relatively good results6, as for the number of killed bacteria. However no study was conducted about the destruction time efficiency. To shorten the
destruction time, lytic phages’ diffusion needs to be maximized.

Our hypothesis is based on the Trojan horse effect: lytic phage released from the inside of the biofilm could kill bacteria faster than lytic phages added on the surface of the biofilm.

Lysogens could be used as Trojan horses. The idea is to introduce them into a growing biofilm and wait for them to be enclosed inside it. Then an antibiotic is used to induce bacteria in lytic cycle, which means phage become virulent.

Since few studies have been realized using lysogens to kill biofilms, we created a mathematical model to predict the optimal destruction time.

RESULTS

Experiment
We grew two types of biofilm:
- TG1 wt biofilms
- TG1 wt biofilms where TG1 lysogens were added 4 hours later.

Three hours later (t induction) we induced the lysogens. The purpose was to show if enclosed lysogens would have perks when it comes to phages release.

We observed that induction of lysogens is significantly decreasing the number of living bacteria in the biofilm (from 10 to 100 fold, fig 2A). We added phages at t induction and we could see that nearly all the cells were lysed by them. We also checked from our control (fig 2B(1)) that ciprofloxacin is killing very little cells (from 0 to 100 cells).

On the other hand we wanted to measure the proportion of bacteria killed within the biofilm considering the different techniques. Adding Ciprofloxacin at t induction (7h) kills 7% of the cells (2B(1)), whereas inducing the lysogens, seven hours after the beginning of the biofilm’s growth, (ciprofloxacin effect taken into account) kills 75% of the biofilm’s bacterial population.

We measured the number of living bacteria in both cases using the CFU method.
Model
We constructed a model to describe the evolution of each population (wt TG1, lysogens, phages and resistant bacteria). The model, shown in Materials and Methods, consists of four differential equations, one for each population.

Figure 3: Model Prediction

Definition: The induction time is the moment when we induce. The minimum time is the moment when there is the smallest amount of bacteria. The destruction time is the duration between the induction time and the minimum time.

Figure 3.A: On this figure we can see the dynamic of the global number of bacteria. After induction, the number of bacteria increases a bit but then decreases until it reaches a minimum (at the minimum time). From that point, bacteria start growing exponentially again because of the growth of remaining lysogens and the formation of resistant bacteria.

Figure 3.B.1: As the induction time increases, the percentage of total living bacteria decreases. The curve seems to present a horizontal asymptote, which means that the longer we wait, the less we gain in efficiency (percentage of living bacteria). R²=0.99

Figure 3.B.2: As the induction time increases, the destruction time decreases. The curve seems to present a horizontal asymptote, which means that the longer we wait, the less we gain in velocity. R²=0.99

Figure 3.C.1: For less than 101 phages, there is no minimum reached (bacteria keep on growing). More than 101 phages, the curve follows a horizontal asymptote (percentage of living bacteria never reaches 0%).

Figure 3.C.2: For less than 101 phages, the destruction time tends toward infinity because there is no minimum time. For more than 101 phages, the destruction time decreases as the number of phages increases.
First, we did a simulation to find the optimal induction time (we fixed the number of phages, \( p \), at 150). In order to find it, we simulated the population dynamics for different induction times. This simulation showed that the more we wait to induce, the smaller is the percentage of living bacteria (fig 3.B.1), and the faster we reach this percentage (fig 3.B.2). However, bacteria start growing again after the minimum time (fig 3.A), because of the formation of resistant bacteria. We also did several simulations for an induction time of 45 min, varying the number of phages released by one bacterium. For less than 101 phages, bacteria keep growing even after induction time. However, for at least 101 phages, a minimum is reached. The more phages are released, the smaller is the percentage of living bacteria at the minimum time (fig 3.C.1), and shorter is the destruction time (fig 3.C.2).

CONCLUSION
Due to the fact that biofilms pose various problems and are difficult to get rid off with common methods, it was proved that lytic phages are a great alternative. Still, different means can be thought of. Our technique, though less efficient, remains very capable, for our experimental conditions. It could be an auxiliary way to explore phage therapy to solve biofilm issue. Regarding our model, we show that the longer we wait to induce, the faster we reach a minimum of living cells. Moreover, this minimum could be reached by releasing more phages per bacterium. This model could be used as a stepping stone.

DISCUSSION
In our work we showed that using lysogens to destroy biofilm could be very effective, and reaffirmed that phages in general are efficient to destroy cells. Thus our method can be seen as an alternative way, considering that the amount of bacteria is significantly reduced after induction and that ciprofloxacin is sparsely harmful to the cells. To assert it stronger, complementary experiments should be done.

In our experiments, we wanted to compare the mixed biofilm with a wt biofilm, but we had obvious technical troubleshooting with the CFU. The control of the wt biofilm is unexploitable (fig 2A, wild type only). However, as the wt and lysogens have the same growth rate in liquid media, we assumed that the wt biofilm is similar to the uninduced biofilm with lysogens. (fig 2A, with lysogens)

Our experiment shows that the use of \( \lambda \)vir is more efficient than inducing lysogens. Nevertheless, we only tested one set of experimental parameters. This is why we used our model to explore other ones.

Although the model shows that the more we wait to induce, the smaller is the percentage of living bacteria, real-life issues do not follow this logic.

However, reducing the number of living bacteria and the destruction time is also possible by increasing the number of phages released per bacterium for a given induction time. This might be done thanks to bioengineering, or by choosing a phage which replicates faster.

Moreover, a kinetic experiment was performed in liquid media, and we do not know if it is the same in biofilms. Testing it could allow us to adjust the model.

It also would have been more accurate to create a 3-Dimensional model to illustrate the fact that our biofilm is not homogeneous\(^4\). Coupled with microscopy imaging, it would describe the dynamics of the four populations more precisely. For the fact that we do not know where the lysogens grow when added to the biofilm: knowing if they grow on the surface or inside the biofilm would give us an insight on whether it is the number of phages released or their location which explains the cells death.

Eventually, a diffusion parameter could be added to the model to illustrate the inducer's propagation. Plus bacteria's size could be taken into account to calculate the meeting rate between a phage and a bacterium (using the mean volume explored by a phage).

Finally, the model should be compared to experimental results to test its validity. It was
not done because the model parameters did not match with the experimental ones.

MATERIALS AND METHODS

Materials
We used E.coli strain TG1, their genotype is: [supE hsdA5 thi Δlac-proAB/F’ (traD36,proA+B+,lacIq, lacZΔM15)]. The phages strains used are the following: *Ur-Lambda phleoR* of genotype stfR+ phleoR (which carries a resistance gene to phleomycin) and *Lambda vir* of genotype cl. We constructed ourselves lysogens. To check the lysogenicity of our potential *Ur-Lambda phleoR* lysogens, we plated our bacteria on LBA and phleomycin (10µg/ml).

We performed our experiments in Cellstar standard Greiner Bio-One 96 micro assay plates using LB media (trypton: 10g/L; yeast extract: 5g/l; NaCl: 10g/l). We used LBA media (trypton: 10g/L; yeast extract: 5g/l; NaCl: 10g/l; Agar : 15g/l) for Petri dishes.

We plated 100 µL of the last three dilutions (10^-6; 10^-5; 10^-4) on LBA plates, and spread them out using the beads technique. We put the plates at 37°C overnight. A specific CFU on LBA complemented with phleomycin, was realized to count lysogens.

Methods
Experiments were conducted in 96 well micro assay plates. Each well was filled with a total volume between 150 and 160 µL. Each condition was done in triplicate.

Our experiment consisted in growing a wt biofilm, introducing lysogens 4h later, and inducing 3h30 after. Controls can be found in Figure 1.

**TG1 wt biofilm growth**
To start the biofilm growth, 10 000 TG1 wt cells, from an overnight culture, were added to 100µL of LB media, in micro assay plates. It was then incubated at 37°C for four hours.

**Lysogen introduction**
Once the wt biofilm was created, 10 000 TG1 lysogens of *Ur-Phleo Lambda* were added in the well. The biofilm was put back in the incubator, at 37°C for three hours and a half.

**Lysogens induction**
To induce the lysogens, we used ciprofloxacin, an antibiotic which triggers the SOS response of the lysogens, thus releasing the phages. The ciprofloxacin concentration used for induction was 2µg/mL.

We put back the micro assay plate in the incubator for one hour at 37°C.

**Stop of the biofilm’s growth**
We emptied micro assay plate, from the supernatant liquid LB remaining over the biofilms, by flipping over the micro assay plate, and dried it with a paper towel.

**Resuspension and plating**
We resuspend each biofilm in 100 µL of LB media. We diluted each well using serial dilution, in order to conduct the Colony Forming Unit (CFU) test.

We plated 100 µL of the last three dilutions (10^-6; 10^-5; 10^-4) on LBA plates, and spread them out using the beads technique. We put the plates at 37°C overnight.

A specific CFU on LBA complemented with phleomycin, was realized to count lysogens.

**Model**
With our model, we aimed at finding the optimal induction time, the one that leads to the lowest number of bacteria, for a given experiment duration.

We have four differential equations that describe the dynamic of four populations from the moment when we add the lysogens cells: wild type bacteria (W), lysogens (L), phage (P) and phage-resistant bacteria (R) which correspond to the new lysogens that are formed after induction (called resistant bacteria because of the fact that a lysogen cannot be infected again). Our system is the following:

$$\frac{dW}{dt} = gr.W(t) - tlys.tinf.W(t).P(t) - tlyt.tinf.W(t).P(t)$$

$$\frac{dL}{dt} = gr.L(t) - h(t).im.L(t)$$

$$\frac{dP}{dt} = h(t).p.im.L(t) + p.L(t) - tlys.tinf.P(t).W(t) - tlyt.tinf.W(t).L(t)$$

$$\frac{dR}{dt} = gr.R(t) + tlyso.W(t).P(t)$$

Our parameters are:
Gr  | 2.28div/h  | Growth rate of wild-type, lysogens and resistant bacteria
---|---|---
Tinf | Depends on Mean volume explored by a phage
Tlys | 0.0001 | Rate of lysogens formed after meeting a lytic phage
Tlyt | 0.9999 | Rate of phages entering a lytic cycle
Im | 1 | Induction rate by the inducer (ciprofloxacin)
p | 150 | Number of phages released by a bacterium
Ti | 0.75h | Induction time

$h(t)$ the a Heaviside function.
The growth rate was measured experimentally, and the mean volume explored by a phage was calculated based on the work of Jun Hu, Kazuhiko Miyanaga, and Yasunori Tanji in their article: Diffusion of Bacteriophages through Artificial Biofilm Models.

**Experimental determination of the growth rate (gr)**
We performed a kinetic to determine the growth rate of wild-type and lysogens. We measured the Optical Density (OD) of our liquid cultures each 10 minutes for 80 minutes.
We found that the growth rates of wild-type and lysogens are the same, equal to 0.030 divisions/minute, meaning 2.28 divisions/hour.

**Calculation of the mean volume explored by a phage (tinf)**
Since our hypothesis is based on the diffusion of phage, we had to define a term to model the spatial dimension of our problem.
We know that during a time $t$, a phage explores on average, a spherical volume of $\frac{4}{3}\pi(\sqrt{Dt})^3$, with $D$ the diffusion coefficient in $m^2.s^{-1}$, according to the laws of diffusion. In our model, $t=0.1$, and represents the step. According to Jun Hu, Kazuhiko Miyanaga, and Yasunori Tanji, $D$ is $2.4 \times 10^{-13} m^2.s^{-1}$. Then, $t_{inf} = \pi \frac{4}{3} (\sqrt{24 \times 10^{-13}} \times 0.1)^3$.

We did a simulation on Scilab for a 5 hours experiment.

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