Spontaneous *Escherichia coli* persisters with week-long survival dynamics and lasting memory of a short starvation pulse

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Abstract

The vast majority of a bacterial population is killed within a time scale comparable to their generation time when treated with a lethal concentration of antibiotics. However, a small subpopulation typically survives for an extended period. To investigate the long-term killing dynamics of bacterial cells we constructed a week-long killing assay and followed the survival fraction of an *E. coli* K12 strain exposed to a high concentration of ciprofloxacin. We found that long-term survivors were formed during exponential growth in both a wildtype and a *relA* deletion strain, with some cells surviving at least 7 days. The killing dynamics showed at least three time-scales, in contrast to the commonly assumed biphasic killing. Furthermore, we observed a surprisingly long memory effect of a brief starvation pulse, which was dependent on *relA*. Specifically, one hour of carbon starvation increased the surviving fraction by nearly 100-fold even after 4 days of antibiotics exposure.

Keywords: Bacterial persistence, Stress response, Exponential phase, Antimicrobials, (p)ppGpp

1. Introduction

Bacterial populations are quickly decimated during a typical antibiotics assault. Within a few generation times, the far majority of cells will be dead. However, it is typically recommended to use extended durations of treatment, ranging from several days to months, prolonging the exposure time of bacterial pathogens to the antibiotic [1]. The WHO is now considering the benefits of shortening the duration of antibiotics administration while still keeping the treatment effective, due to concerns of increasing antibiotic resistance occurring as a consequence of increased exposure [1]. To find the optimal treatment duration, one needs to understand the killing dynamics of bacteria when exposed to antibiotics, especially the bacterial cells surviving for longer times. The long-term survivors are typically referred to as persister cells, a subgroup of cells that survive antibiotics for an extended period compared to the average of the population, but have not acquired mutations that make them resistant to the antibiotic [2, 3]. Most research on persister cells is done within the well-defined conditions of the laboratory, but despite these strongly simplified conditions, and more than seventy years of research, laboratory persisters are still far from understood [4, 5].

One pending question is whether persisters form spontaneously during the exponential growth phase. It was repeatedly shown that stress-triggered persisters are formed in high numbers during the stationary phase, but research on spontaneous persister formation during the exponential phase is sparse [4]. The research has mostly been confounded by a lack of careful attention to the presence of stationary phase cells carried over from the starter cultures, which artificially elevated the persister fraction of exponential cultures [5, 6, 4]. One carefully executed study showed that no *E. coli* persister cells were formed during fast exponential growth in rich medium [7], whereas other studies merely showed reduced levels during exponential growth [5]. A benefit of analysing the exponential growth phase is the well-defined physiology of this state [8, 9]. This makes it possible to vary the growth physiology in a controlled manner, especially by varying the growth rate through culturing bacteria in media of different nutrient quality. It was previously shown that the bacterial growth rate strongly correlates with the death rate during the initial period of killing with beta-lactams [10, 11, 12]. This poses the additional questions of how the growth rate at the time of antibiotics exposure affects the short- and long-term killing dynamics.

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The current standard for persister identification at the population level is a biphasic killing curve, where two time-scales are identified in the time-kill curves [4]. Persisters are identified as a subpopulation with a second, slower killing rate than the rapid death rate of the primary population. If only two time-scales are present in the killing dynamics, the population survival time can be extrapolated from the second slow killing rate. In fact, one study mentions a tail heavier than the biphasic model, but discards these long-term survivors as contamination in the persister assay [13], whereas another study identifies a powerlaw tail in the short-term killing dynamics [14]. However, there is not much research on the long-term survival of the antibiotics-tolerant subpopulation. Despite the recommendations for week-long treatments by the WHO [1], most *in vitro* lab research on persisters is carried out for approximately three to five hours [15, 7, 5], though some studies increase the exposure time to 24–50 hours [2, 16, 17, 3]. The investigation of long-term survival beyond the typical five-hour persister assay might reveal new insights into bacterial killing dynamics.

Lastly, the molecular mechanism(s) of persister formation is still unknown [18, 12]. Many intracellular components have been proposed [19, 15, 20, 21, 22, 23], but so far no single mechanism convincingly explains persister formation. In fact, bacterial persistence presents as a very complex and diverse problem, where the survival fraction could be composed of different subpopulations. It is known that stationary phase cultures contain a greater persister fraction than exponentially growing cultures [5] and that the stationary phase is typically associated with starvation stress, despite the multitude of different physiological states it might refer to [24]. Furthermore, the second messenger (p)ppGpp that accumulates during starvation responses was frequently shown to correlate positively with persistence formation [17, 25, 5, 21].

The present study investigates persisters in the balanced exponential growth phase. It deals with whether *E. coli* forms spontaneous persisters in the exponential phase, their dependence on the growth rate, how long they survive and how their formation relates to the secondary messenger (p)ppGpp. We followed the long-term survival of an *E. coli* strain exposed to a lethal concentration of ciprofloxacin for one week. The growth rate of the *E. coli* strain was modified in two different ways. First of all, a knockout strain was constructed in the wildtype background, removing the gene *relA* and, thus, introducing a (p)ppGpp synthesis deficiency. Secondly, the growth medium was varied by growing the two strains with one of two different carbon sources, leading to different growth rates. Lastly, we compared these killing dynamics with and without a carbon source downshift prior to the killing assay. This starvation pulse had a considerable influence on the persister formation and had a very long memory effect in the survival with at least four days of significantly enhanced persister levels.

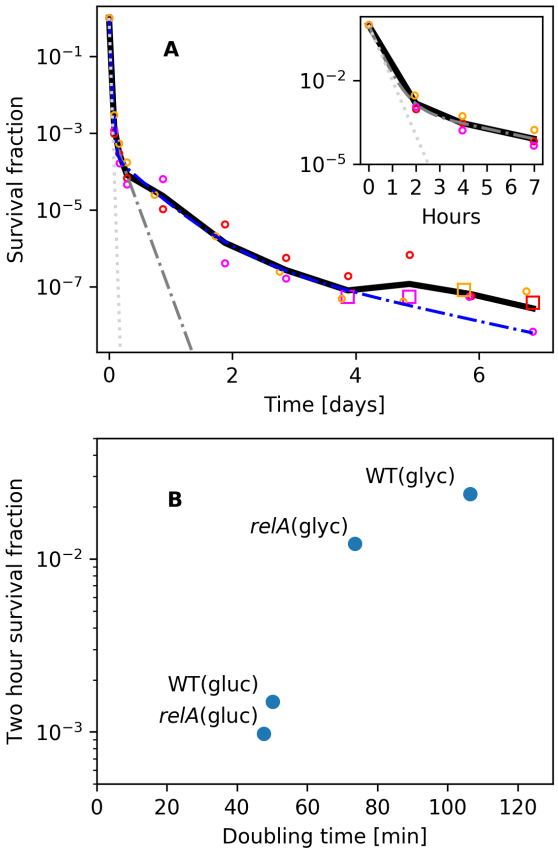

Figure 1: Killing dynamics of exponential phase *E. coli* persisters. (A) Killing dynamics for exponential phase persisters. The bacteria were grown in glucose minimal medium. All three biological replicates are shown for each datapoint. The black line is the geometrical mean. The grey dash-dotted line is a biphasic fit to the initial phase of killing, and the lightgrey dotted line shows the largest exponential slope from the biphasic fit. The blue line shows a triphasic fit to the first four days of killing. (B) Survival fraction after two hours of ciprofloxacin treatment compared to the growth rate prior to antibiotics treatment.

2. Results

Long-term persister assay of exponentially growing cells

To investigate whether long-term persister cells form during exponential growth in glucose minimal medium,

we treated balanced cultures of *E. coli* K-12 with ciprofloxacin and monitored the killing dynamics for one week of antibiotics treatment. Balanced growth was obtained by culturing the cells for more than twenty doublings in the target medium at 37 °C, keeping the cell density of the culture below an OD_{436} of 0.3 by repeated back-dilutions. Cultures were then treated for a week with 10 $\mu g/mL$ ciprofloxacin and their killing dynamics were monitored by repeated platings of culture aliquots on antibiotics-free growth medium (See methods for detail).

Week-long survival of *E. coli* persisters were formed during exponential balanced growth in glucose minimal medium, as seen in Fig. 1A. There was a fast initial killing, with a slower killing rate already after two hours, as seen in the inset in Fig. 1A. The second phase of killing seemingly extended from two to seven hours of killing. Indeed, a biphasic curve (two exponentials) can reasonably fit the first 7 hours data as shown as a grey line (Fig. 1A inset). However, for longer times, this fit significantly underestimates the survival time of the bacterial population (Fig. 1A). In other words, there is a "third phase" of slow killing, extending from seven hours to four days of killing. Lastly, from day five to day seven, the cells were killed at a very slow rate, however, this part of the data is less reliable due to small numbers of surviving colonies.

To identify the various phases in a quantitative manner, the data was fitted with a sum of exponentials, and the appropriate number of exponentials was chosen with a χ^2 test (see SI Appendix section 2). The test rejects a double exponential as a good fit, but accepts the hypothesis of a triple exponential. The long-term killing dynamics thus had more than two time-scales, in fact, the third scale was much more dominant in the killing dynamics by representing several days of survival, whereas the first two phases were over in less than ten hours.

The exponential phase growth rate determines many aspects of bacterial physiology, including the macromolecular composition [8, 26]. The growth rate at the time of antibiotics exposure has previously been linked to short-term survival of antibiotics [10, 11], and could also affect long-term survival. For that reason, the long-term killing assay was repeated with glycerol as the carbon source, which strongly affected the wildtype growth rate. In glucose minimal medium, the wildtype doubling time was 50±1.4 min, while it was 106±3.0 min in glycerol minimal medium. This difference had an impact on the initial phase of killing for up to seven hours, as shown in the Fig. 2A inset. However, long-term survival was not significantly affected by the growth rate.

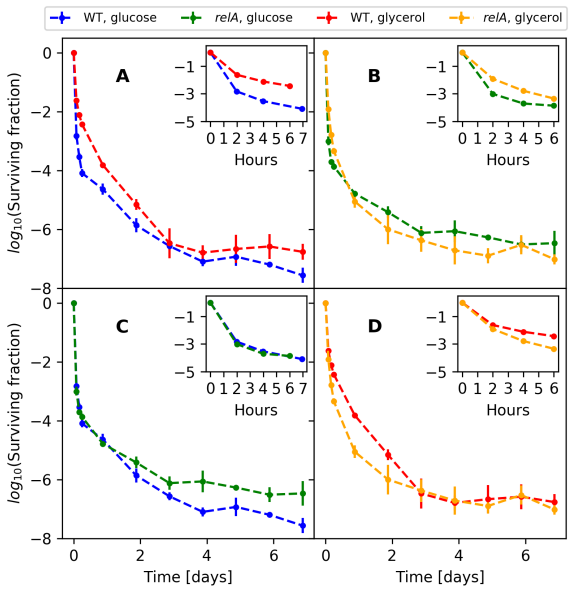

Figure 2: Killing dynamics in different medium with and without the *relA* gene. Each line represents three biological replicates. (A) The wildtype killing dynamics in either glycerol or glucose minimal medium. (B) The Δ*relA* killing dynamics in either glycerol or glucose minimal medium. (C) Killing dynamics in glucose minimal medium for comparison between the wildtype and the Δ*relA* strain. The Δ*relA* mutant had more survivors than the wildtype from day 4 and later, but there was no statistically significant difference at any of the timepoints (SI Appendix section 2). (D) Killing dynamics in glycerol minimal medium for comparison between the wildtype and the Δ*relA* strain.

In fact, in two of the three biological replicates concerning wildtype survival in glycerol, almost no survivors were observed after 3 days of killing (see SI Appendix section 1). However, more cells survive in glycerol than in glucose for up to two days, as seen in Fig. 2A. The wildtype killing dynamics in glycerol had more than two time scales, with a best fit of four separate phases of killing (SI Appendix section 2).

Deletion of relA affects the killing dynamics

In glycerol minimal medium, the steady state (p)ppGpp level of the wildtype strain is higher than in glucose minimal medium [27]. Because the (p)ppGpp level has been frequently associated with persister formation, we next aimed at investigating the effect of the (p)ppGpp level on long-term survival. *E. coli* encodes the primary (p)ppGpp synthetase RelA and the secondary (p)ppGpp synthetase SpoT, the latter of which is bifunctional as a (p)ppGpp hydrolase. The nutrient-dependent steady-state growth rates are inversely related to the concentrations of (p)ppGpp, both for *relA*$^+$

an *relA*$^-$ strains [27], and in many media, the (p)ppGpp level of *relA*$^+$/*relA*$^-$ strain pairs are indistinguishable. However, in low energy carbon sources like glycerol or acetate, SpoT produces insufficient (p)ppGpp to suppress the growth rate when RelA is missing, leading to an enhanced growth rate of the RelA mutant strain relative to the wildtype [27]. We constructed a Δ*relA* mutant to clarify the role of (p)ppGpp in the killing dynamics.

As expected, the difference between the growth rate in glucose and in glycerol minimal medium was smaller for the Δ*relA* strain, with a doubling time of 47±1.5 min in glucose minimal medium and 74±1.6 min in glycerol minimal medium. The short term survival under antibiotics exposure was also correlated with the growth rate in the Δ*relA* strain (Fig. 1B inset), and in glucose this strain had initial killing dynamics similar to the wildtype (Fig. 2C inset).

The Δ*relA* mutant also formed long-term survivors in both glucose and glycerol minimal medium with more than two phases of killing (Fig. 2B, see SI Appendix section 2 for statistical analysis). In glucose, the early time killing dynamics of the Δ*relA* mutant were very similar to that of the wildtype (Fig. 2C), while in glycerol, there was a significant difference in the initial phase of killing between the wildtype and the Δ*relA* mutant up to one day (Fig. 2D, see SI Appendix section 2). Interestingly, the long-term survival of the Δ*relA* mutant at later times was comparable to that of the wildtype strain in both media.

Short downshift prior to the antibiotic application affects the long-term persistence of wildtype cells in glucose minimal medium

The killing dynamics dependence on both the *relA* gene and the carbon source indicates that the level of the second messenger (p)ppGpp plays a role in persistence formation in balanced growth. We then wondered if a short pulse of starvation, which is expected to give a short spike in the (p)ppGpp level in the wildtype strain, but not in a RelA strain [28], could affect the killing curve. The idea was to investigate whether a short perturbation to the exponentially growing cells prior to the killing assay, could have a long-lasting effect on the killing dynamics.

Cultures in balanced growth were filtered into growth medium without a carbon source and starved for 1 hour, before the carbon source was replenished. Antibiotics were added simultaneously with the carbon source replenishment (Fig. 3, see methods for details.). Remarkably, the short nutrient downshift prior to the addition of antibiotics had long-term effects on the killing dynamics. This was especially true for the wildtype strain grown in glucose minimal medium, where there was a significant difference between the starved and the unstarved cultures for up to four days, as seen in Fig. 4A. The difference was abolished by removing *relA*, as seen in Fig. 4B; the Δ*relA* strain only exhibited increased survival for the first six hours following a downshift. As such, the long-term memory of the starvation pulse is seemingly a *relA*-dependent effect. However, the Δ*relA* strain generally had more long-term survivors than the wildtype and the downshift brought the wildtype survival fraction to a level similar to the Δ*relA* strain. The long-term effects of a downshift are absent in glycerol minimal medium, which is seen in Fig. 4CD.

3. Discussion

We expanded the understanding of bacterial killing dynamics with a new long-term persister assay. The use of minimal medium facilitated the formation of long-term persisters during exponential growth. This is opposed to the commonly held belief that exponential phase cultures do not contain persisters, which was concluded based on growth and killing in rich medium [7]. Spontaneous persisters do form during the exponential growth phase, both in glucose and glycerol minimal medium, and in some cases they survive at least one week. This long-term survival does not require *relA*, although the residual (p)ppGpp synthesized by SpoT is likely necessary. In fact, there is an increase in long-term survival of the Δ*relA* mutant in glucose.

We have shown that a one-hour starvation pulse prior to the addition of the antibiotic affects long-term survival. This finding is consistent with a previous study of a temporal nitrogen downshift prior to antibiotics treatment, which was shown to elevate the persister level at 24 hours in a *relA* dependent manner [29]. Our study demonstrated that the memory can be remarkably long-lasting, as one-hour carbon starvation gave an increase in survival for at least 4 days in the wildtype strain grown in glucose medium. The molecular mechanism underlying this long-term memory is yet to be investigated, but in all likelihood it is linked to the abrupt RelA-mediated rise in (p)ppGpp upon starvation, since the starvation-pulse effect on long-term survival was abolished in the Δ*relA* mutant. In further support of this hypothesis, there was no long-term effect when glycerol was used as the carbon source, which could be due to the high basal level of (p)ppGpp in glycerol relative to glucose minimal medium [8]. The sensitivity of the survival fraction to the rather short perturbation may be related to the theoretical prediction that a small perturbation can have a major impact on the occurrence of

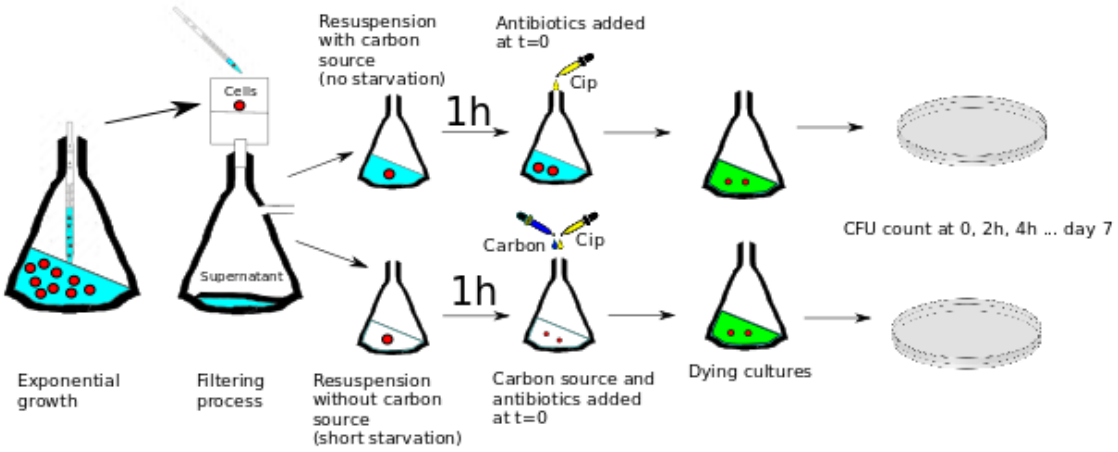

Figure 3: The experimental setup for the long-term persistence assay. Cells were in balanced exponential growth prior to the killing assay. A part of the culture is filtered and then resuspended in medium w/o the carbon source. They are growing (or not growing) like this for one hour. The first sample is taken and instantaneously the carbon source is added to the flask without the carbon source, and at the same time the ciprofloxacin. Then, samples were taken at 2,4,6/7, 21+24·n hours after the antibiotic addition for $n \in [0; 6]$. The samples were washed and plated on agar plates containing the target medium.

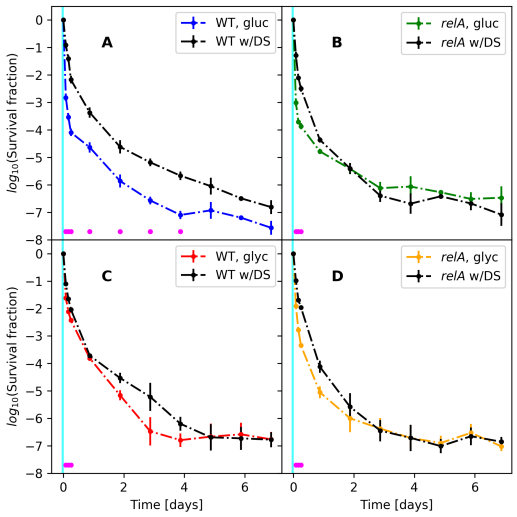

Figure 4: The effect of a short starvation-pulse on the survival dynamics. All strains and conditions are shown with and without the downshift. The black line is with the downshift, the colored line is without the downshift. The magenta dots represent statistically different datapoints as tested by an unequal variances t-test. The cyan interval illustrates the downshift period prior to the killing assay. (A) The wildtype in glucose. A downshift in glucose significantly enhances wildtype survival for four days and seems to be increased for up to all seven days. (B) The Δ*relA* mutant in glucose. (C) The wildtype in glycerol. (D) The Δ*relA* mutant in glycerol.

rare discrete expression patterns [30]. This observation also alerts us that small perturbations in the experimental procedure may strongly affect the result of persister assays.

This study shows that the long-term killing of *E. coli* in ciprofloxacin is not adequately described by biphasic dynamics. At least three phases of killing were present in the data. Thus, despite the emphasis on a biphasic behaviour to define persistence [4], we propose a third, or even fourth, phase of killing that may even be more clinically relevant. The presence of additional phases also means that the population survival time will be underestimated by predictions from the biphasic killing assumption. Indeed, it is not sufficient to measure killing dynamics for only five hours and then extrapolate the population survival time from there. The presence of several phases in the killing dynamics begs the question if a more extended concept should replace the simplified concept of bacterial persistence.

The population growth rate had a small effect on persistence formation, where only the initial killing rates were correlated with growth differences. In shorter persister assays, a difference in growth rate, such as between different mutants, might strongly confound results when analyzing persistence fractions. These differences seem smaller and less relevant in later phases of killing.

This investigation of long-term killing dynamics has added to the concept of bacterial persistence as a complex phenomenon. During long-term antibiotics treatment, different mechanisms could account for bacterial survival on different timescales (an hour, a day, a week), although the (p)ppGpp level at the time of initial exposure to the antibiotic seems to be important in all cases. As such, persistence seems to be a time-dependent phenomenon, where different survival mechanisms account for different bacterial life spans.

4. Methods and Materials

Long-term killing assay

A single colony from a plate was incubated overnight in the target medium (MOPS minimal medium with either glucose or glycerol as the carbon source [31]. See the SI Appendix section 1 for the recipe). The overnight culture was diluted $1:10^7$ in 10 mL target medium in a 100 mL Erlenmeyer flask. The flask was continuously shaken at 160 RPM in a 37°C room. Hours later, the culture was diluted further, at least $1:10^2$, reaching a dilution of at least $1:10^9$. Once the OD_{436} reached a detectable level, at least 5 samples were measured at different timepoints to establish a growth rate. The OD_{436} was consistently kept below 0.3. A part of the culture, typically around 20 mL, was filtered, and the cells on the filter were resuspended in 40 mL of the target medium (w/o carbon source) in a 300 mL Erlenmeyer flask. The medium volume never exceeded 14 percent of the flask volume. The starvation was verified by measuring OD_{436}, to confirm either increase in biomass (control) or no growth (starving culture). See the SI Appendix section 1 for confirmation of the downshift. After one hour of starvation, a sample was taken immediately before ciprofloxacin was added along with the carbon source, that was replenished to end the downshift. Samples were taken at times 2,4,6/7, $21+24\cdot n$ hours for $n \in [0;6]$. The samples were put on ice for a few minutes and then centrifuged for ten minutes at 4°C at 10,000g. The supernatant was removed and the cell pellet was resuspended in room temperature MOPS buffer with no supplements. The sample was diluted appropriately, never more than $1:100$ per step, corresponding to 10 μL in 990 μL. The sample was plated with 200 μL per plate on minimal medium plates containing the target medium. The plates were kept at 37°C for at least one week and all colonies were counted. The whole experiment is illustrated in figure 3. The strain MAS1081 (MG1655 *rph*$^+$ *gatC*$^+$ *glpR*$^+$) was used as the wildtype [32]. The Δ*relA*, MAS1191, is MAS1081 made Δ*relA*251 :: *Kan* by P1 transduction from CF1651 [33] followed by selection on kanamycin.

Controls

After each finished experiment (7 days), 200 μL of culture, still containing antibiotics, was spread on a plate with the target medium. This was left at 37°C for at least 7 days, to detect if there was a growing resistant culture in the flask. In addition, the activity of the antibiotics in the culture was tested by dropping 20 μL on a lawn of growing *E. coli*.

Analysis of time-scales in the killing dynamics

Time-scales in the killing dynamics were statistically identified by fitting a sum of exponential functions to the data. The model with the least number of parameters, that could not significantly be rejected, was then chosen [34]. The functional form of the models was a sum of exponential functions. The number of exponential functions in the sum corresponded to the number of time-scales. A biphasic killing curve would for example be well fitted by the sum of two exponentials. The χ^2-test for goodness of fit is used for identifying the appropriate model using the Minuit minimization software [35, 36] (see the SI Appendix section 2 for specifics of the analysis).

Statistical analysis

All killing curves are based on three biological replicates. The mean is determined as the mean of the logarithmic values, which corresponds to the geometric mean. This is done to get a more adequate mean-value representation in log-space. The uncertainties are also calculated as the standard deviations of the log transformed values. Whenever a datapoint had the value zero, which happened frequently, that value was replaced with the detection limit, to get a sensible value in log-space. An unequal variance t-test was used to determine significant differences between two datapoints at the same timepoint.

Acknowledgments

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