

# An Experimental Framework for Quantifying Bacterial Tolerance

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**ABSTRACT** Antibiotic tolerance and persistence are often associated with treatment failure and relapse, yet are poorly characterized. In distinction from resistance, which is measured using the minimum inhibitory concentration metric, tolerance and persistence values are not currently evaluated in the clinical setting, and so are overlooked when a course of treatment is prescribed. In this article, we introduce a metric and an automated experimental framework for measuring tolerance and persistence. The tolerance metric is the minimum duration for killing 99% of the population,  $MDK_{99}$ , which can be evaluated by a statistical analysis of measurements performed manually or using a robotic system. We demonstrate the technique on strains of *Escherichia coli* with various tolerance levels. We hope that this, to our knowledge, new approach will be used, along with the existing minimum inhibitory concentration, as a standard for the in vitro characterization of sensitivity to antimicrobials. Quantification of tolerance and persistence may provide vital information in healthcare, and aid research in the field.

## INTRODUCTION

The emergence and spread of resistant pathogens as a result of antibiotic selective pressure is a major concern in modern healthcare. Resistance is typically achieved by mutations that reduce the activity of an antibiotic, for example by decreasing drug binding to the target. The level of resistance is quantified for each bacterial isolate by the minimum inhibitory concentration (MIC), which is the minimal concentration required to prevent bacterial growth (1). The MIC is routinely measured in hospitals, and is a crucial factor in decisions regarding antibiotic treatment.

Tolerance, on the other hand, is a poorly characterized phenomenon, and is seldom taken into account explicitly in healthcare. Unlike resistance, which is an increase of the drug concentration in which the bacteria can grow indefinitely, tolerance is an extension of the period of time that bacteria can survive in lethal concentrations of an antibiotic (many times the MIC) before succumbing to its effects (2). There are several mechanisms possibly underlying tolerance, recently reviewed elsewhere (3,4). For example, tolerance to  $\beta$ -lactams may be achieved by a decreased rate of cell-wall building (5), and tolerance to fluoroquinolone results from a decreased rate of DNA replication (6). It is note-

worthy that many antibiotics target essential growth processes and, as a result, many tolerance mechanisms cause a decrease in growth rate.

It has been observed that tolerance can be heterogeneous in bacterial populations, namely that subpopulations with different tolerance levels may coexist within the same clonal population, a phenomenon termed “persistence” (7), or more precisely “time-dependent persistence” (4). For example, when only a small subpopulation of transiently dormant bacteria are present in a bacterial population, they will be able to persist for a longer period of time under antibiotic treatment, compared to the majority of fast growing bacteria. This effect is apparent in a biphasic time-kill curve (8), and may be viewed a special case of tolerance. Other survival strategies exist, including different forms of persistence (4,9,10). These are not discussed here, as they are not tolerance-based, although some may be of similar clinical relevance.

Although tolerance may seem less advantageous than resistance, this is only true when long-term exposure to a drug is considered. Under changing conditions, however, tolerance may have a strong advantage, which could have a profound impact on the evolutionary outcome (11). For example, it has been shown that the extension of lag-time, namely the prolonged time to resumption of growth after starvation, evolves fast under intermittent antibiotic treatments and confers high tolerance, before any sign of

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resistance appears (12). Such increased survival has been shown to provide a window of opportunity for the population, facilitating the evolution of resistance (13,14). Moreover, tolerance may provide increased survival under treatment with antibiotics from different classes, as long as they target growth processes. From an immediate clinical point of view, the few surviving tolerant bacteria, those that can outwait antibiotic treatment, may be sufficient to restart an infection once treatment is stopped (15).

Although research into tolerance is ongoing, definitions and measurement techniques are limited (3,4,16). The standard method used to evaluate tolerance is by performing time-kill measurements. In this method, bacteria are exposed to an antibiotic, and viable CFUs are plotted against time (Fig. 1). When the killing is strictly exponential, the killing rate can be used to quantify tolerance. However, time-kill curves rarely fulfill this criterion (Fig. 1), and therefore the killing rate cannot be utilized to compare different strains and conditions. Instead, we have defined a simple timescale parameter to characterize tolerance, much like the MIC is a concentration-scale characterization of resistance (1). We termed this parameter the minimum duration for killing a certain percentile of the population (MDK), with the percentile added as an index—thus,  $MDK_{99}$  is the minimum duration for killing 99% of the population;  $MDK_{99.9}$  is the minimum duration for killing 99.9% of the population, etc. A comparatively high MDK suggests that bacterial killing requires more time, i.e., corresponds to

high tolerance. The tolerant strain plotted in Fig. 1 (blue) evolved from the ancestral strain (green) under intermittent ampicillin treatment, and acquired a mutation leading to a long lag. The mutation had no effect on the MIC (12). The high tolerance of this strain is apparent from the slower killing, which is quantified by the 10-fold increase in the  $MDK_{99}$ , in comparison to that of the ancestral strain (Fig. 1).

Time-kill curves, however, are labor intensive and are rarely performed in the clinical setting. Therefore, we present, to our knowledge, a new method to directly quantify tolerance, without performing time-kill curves, and a more formal definition of the metric used to quantify tolerance, namely the MDK. We propose that the MDK metric be implemented in hospital clinical microbiology labs. This would enable efficient classification of bacterial strains as tolerant, resistant, or persistent (4,17), and thereby provide a typical timescale for clinical implementation of effective treatment of tolerant strains.

## MATERIALS AND METHODS

### Media, chemicals, and growth conditions

Unless otherwise specified, measurements were conducted at 37°C in LB Lennox medium supplemented with 6.25 µg/mL Chloramphenicol.

KLY-derived strains were grown overnight and stored at -80°C with 15% glycerol.

MGY-derived strains were grown in M9 Minimal Salts, 5× (Difco M9; Becton Dickinson, Franklin Lakes, NJ) 0.1% casamino acids, and kept at -80°C with 15% glycerol.

All strains were diluted to least four orders of magnitude before inoculation (as required to reach 1000 CFU/well in the  $MDK_{99.9}$  test or 100 CFU/well in the  $MDK_{99}$  test).

### Robotic setup

A Freedom EVOware 75 base unit (Tecan, Männedorf, Switzerland) was enclosed in a laminar flow hood equipped with a HEPA filter. An 8 Plus 1 liquid handling arm (Tecan) and a RoMa-3 EVO 75 arm (Tecan) were used for pipetting and plate handling. Plates were incubated in a Storex 40 Incubator (Liconic, Woburn, MA) with a shaking option, and culture was kept at 2–3°C on a chill/heat plate (Torrey Pines Scientific, Carlsbad, CA).

### Detailed protocol for measuring MDK

The protocol proposed here for measuring tolerance may be automated easily (as we have done), and is a better quantitative indicator of tolerance than existing methods, as will be explained at length in the Results. The protocol exposes populations of bacteria to different concentrations of antibiotics for varied time periods, and the presence or lack of survivors (regrowth) is used to determine the MDK.

**Preparation.** A 96-microwell plate is filled with antibiotics in concentrations decreasing exponentially with the column. The final column is left antibiotic free, as a control for growth. It should be noted that if the MIC is known (approximately), concentrations below and around it need not be measured. Concentrations should typically reach at least 20× MIC. If the MIC can only be estimated, even up to an order of magnitude, it is advisable to spread concentrations over a wider range, to reach the range where killing is concentration independent.

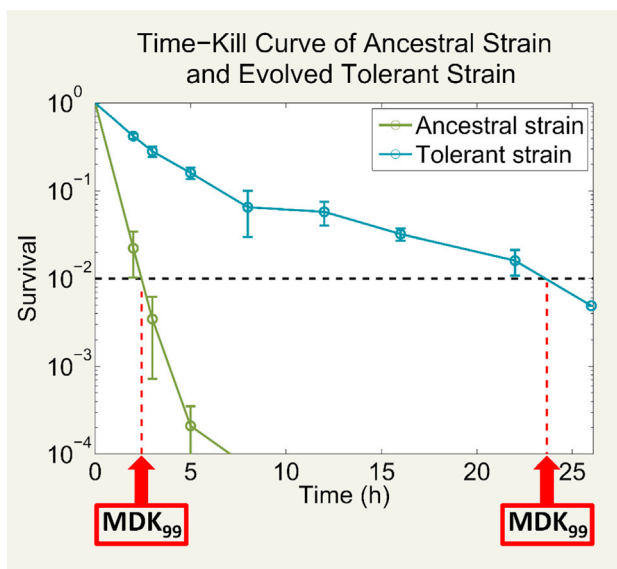


FIGURE 1 Shown here are time-kill curves under ampicillin treatment of two strains with different tolerance levels. The tolerant strain (*tbl5a*) was evolved from the ancestral strain (KLY) under intermittent ampicillin exposure (12). The MIC of both strains is the same, but the tolerant strain is killed at a significantly reduced rate. The slower killing is reflected in the  $MDK_{99}$ , i.e., the time to kill 99% of the population, or Survival =  $10^{-2}$ . Experimental data taken from Fridman et al. (12). To see this figure in color, go online.

Bacteria inocula are diluted to the concentration corresponding to the MDK being measured, so that treatment durations longer than the MDK (time) will result in eradication of the population without regrowth. For example: to determine the  $MDK_{99}$ , 100 bacteria per well are required; to determine the  $MDK_{99.9}$ ,  $10^3$  bacteria should be added to each well; etc. The mean number of bacteria in the inocula,  $N$ , is evaluated by serial dilution and plating.

To maintain viability and metabolic state of the bacteria over time, bacteria are stored in NaCl solution at 2–3°C. We verified that these conditions do not alter the level of tolerance in our strains. Note that, because the physiological state of the bacteria is often key to their tolerance, the details of the preparation of the bacterial culture should be carefully determined, depending on the form of tolerance to be measured. For example, tolerance-by-lag, measured in this work, depends on the lag time of the bacteria and therefore on the growth conditions of the culture before the MDK measurement; cultures grown without adequate shaking, or for too short a time, do not reach a uniform metabolic state. Standardized conditions were chosen, to ensure the entire population reaches stationary phase (Appendix A in the [Supporting Material](#)). Measurement of tolerance by slow growth would also depend upon the growth conditions before the measurement but, unlike tolerance-by-lag, would be best carried out from a strictly exponential culture, such as a chemostat culture.

**Inoculation-incubation cycle.** The plate is inoculated, one row at a time, at set time intervals, and returned to incubation and shaking at 750 rpm. If the MDK is not known at all, a wide exponential timescale should be used. Subsequent assays with a narrower range of inoculation times may be required for accurate MDK determination.

Plates should be allowed to reach 37°C before the first inoculation.

**Antibiotic wash.** Once inoculation and incubation of all rows have been concluded, it is necessary to wash away the antibiotic remains. In the case of ampicillin,  $\beta$ -lactamase is added to all wells at a final concentration of 0.5 unit/mL. Alternatively, two spin-downs are required to reach sub-MIC antibiotic levels in all wells. Each spin-down is 10 min at 1200 g to minimize stress on surviving cells. This procedure is repeated twice, as each spin-down is equivalent to a 10–20-fold dilution, and the highest antibiotic concentration used may be as large as  $100\times$  MIC. In this work, surface-treated plates with U-shaped wells were used (Cat. No. 163320; Thermo Fisher Scientific, Waltham, MA). A model No. 5810R centrifuge (Eppendorf, Hamburg, Germany) was used with a multiwell-plate adaptor, and supernatant was discarded manually. The centrifugation was performed at 10°C, for historical reasons.

**Final incubation and results.** Finally, the plate is incubated overnight with shaking (longer incubation periods may be required for slow-growing strains). Results may be read directly from the plate, as will be discussed, by looking at the high-concentration end, and determining the time at which the treatment becomes too long for the survival of even a single bacterium.

## Determination of MIC and growth rate

MIC was determined by inoculation of bacteria into a 96-microwell plate containing growth medium with different concentrations of antibiotic in different wells, and incubation overnight (1) (24 h for high-tolerance strains).

Maximal growth rate was estimated by measuring the optical density of batch culture over time, although an order-of-magnitude estimate is sufficient for the purpose of this test (Appendix B in the [Supporting Material](#)).

## RESULTS

### The MDK as a measure of tolerance

We now begin with a brief formal definition of the MDK, which captures the typical time required to kill a bacterial population. The MDK can be defined using the survival

function  $S(C,t)$ , which is the fraction of surviving bacteria after antibiotic exposure at a concentration  $C$  and duration  $t$ , and may be visualized as a surface (Fig. 2 a). Various survival functions have been proposed for capturing the effect of bactericidal antibiotics, either from phenomenological or molecular models. The definition of the MDK is independent of the specific model function, as long as the antibiotic's efficacy reaches saturation at sufficiently high concentrations. This is true for many antibiotics, and is implicit in  $E_{\max}$  models, which are commonly used for mathematical description of time-kill curves (18–20). Thus, at high enough concentrations,  $S(C,t)$  is weakly concentration dependent. For antibiotics that do not fulfill this property (10), the MDK is not relevant. The contour plot of the surface in Fig. 2 a is shown in Fig. 2 b, and the contour lines

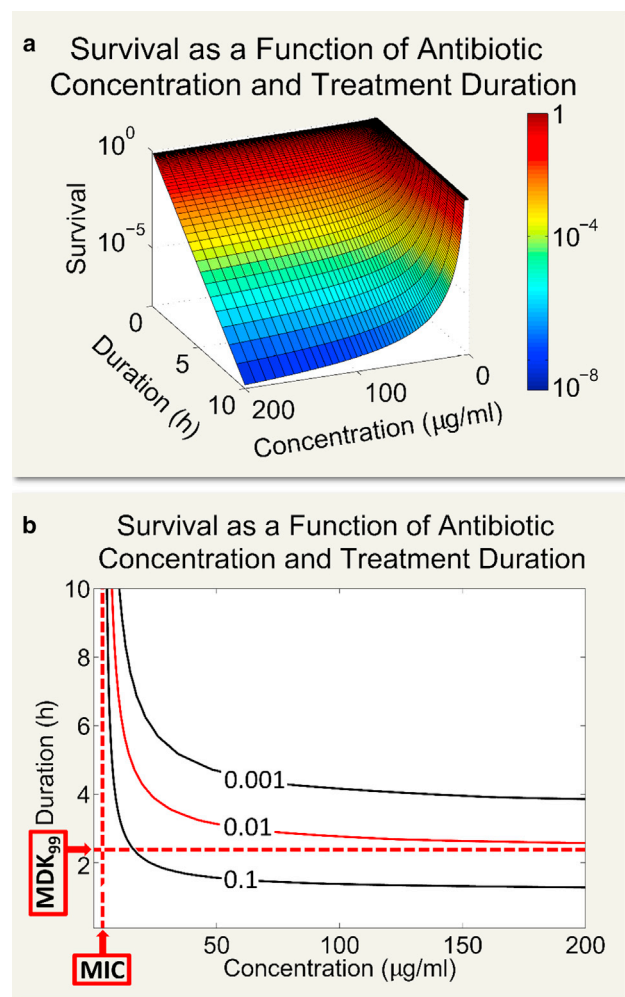


FIGURE 2 Given here is a Zhi model (19) function for survival as a function of antibiotic concentration ( $C$ ) and treatment duration ( $t$ ). Survival is shown both as a surface plot and a contour graph. Note that the Survival has been cut off at  $S(C,t) = 1$ . (a) Given here is Survival as surface plot. The height coordinate of each square represents the survival probability for a treatment of duration  $t$  and concentration  $C$ . (b) Given here is Survival as contour plot. The  $MDK_{99}$  is the horizontal asymptote (high concentration) of the contour line  $S(C,t) = 0.01$ . To see this figure in color, go online.

display both vertical and horizontal asymptotic behavior. The  $MDK_{99}$  is the horizontal asymptote of the contour  $S(C, t) = (1/100)$  (high concentrations), and the vertical asymptote is the MIC (long treatment durations).

Resistance may be described as a shift in the MIC to higher drug concentration. Tolerance can be characterized in a similar manner, by an increase in the MDK ( $MDK_{99}$ , for example), corresponding to survival under longer exposure to antibiotics.

### Automated measurement of the MDK

To measure the MDK without generating the entire time-kill curve, we developed an automated protocol based on multiple probabilistic measurements. We implemented the protocol by an automated robotic procedure, but it may also be performed manually.

We focus initially on  $MDK_{99}$  measurement, namely the time required to kill 99% of the population. By exposing many parallel populations of  $\sim 100$  bacteria each to different concentrations for different durations, it is possible to extract the  $MDK_{99}$  by observing the overall outcome, i.e., whether there were any survivors or not after the antibiotic was removed. This is reminiscent of the method of most probable number (21), where bacteria are serially diluted to concentrations above and below one bacterium (on average) per isolated growth medium, and then observation of the growth/no-growth pattern allows determination of CFU in the original culture. Our protocol for measuring the  $MDK_{99}$  consists of the following steps (Fig. 3). First, a 96-microwell plate is prepared, in which the concentration of antibiotic varies exponentially with the column (Fig. 3 a). Antibiotic containing rows are inoculated with bacteria at different preset times (see [Materials and Methods](#); Fig. 3, c and d). Inoculation times are set so that all rows end their respective treatment at the same time. Once incubation is concluded, the plate is spun down to terminate the antibiotic exposure by washing away antibiotic remains and resuspending in fresh medium. The result of this protocol is a grid of 96 different concentration-duration pairs, to each of which  $\sim 100$  bacteria have been exposed. The plate is then returned for overnight incubation. Empty wells indicate killing of  $>99\%$  of the population, because growth in the well would imply that at least one bacterium, out of the 100 at  $t = 0$ , survived the treatment (see Appendix A in the [Supporting Material](#)). The interface between the growth and no-growth areas is therefore the contour  $S(C, t) = (1/100)$ . For most antibiotics, the effectiveness of the drug reaches saturation at high concentrations (18), as mentioned. This can be seen in Fig. 3 f, where at high enough concentrations, the pattern reaches a plateau, indicating that the dynamics are weakly dependent on concentration in this regime. This is the regime by which tolerance is defined. A rough evaluation of  $MDK_{99}$  can be read directly from the plate—

it is the treatment duration at which the plateau forms (*dashed red line* in Fig. 3, e and f). The experimental evaluation for the strain shown in Fig. 3 f is  $\sim 2.5$  h, in agreement with the time-kill curve of the same strain (Fig. 1). A more precise evaluation of the  $MDK_{99}$  may be achieved using the entire growth/no-growth pattern, as explained below.

### Maximum likelihood estimation of MDK

The span of concentrations ( $C$ ) and times ( $t$ ) in the experimental protocol allows for the direct visualization of the  $S(C, t) = 0.01$  curve and a rough estimate of the asymptotic behavior. The  $MDK_{99}$  can be directly (albeit, roughly) assessed by looking at the duration of exposure that prevents growth at high concentrations. For example, for the strain in Fig. 4 a (KLY), this duration is  $\sim 3$  h under ampicillin. This semiquantitative estimation can be improved by using a more precise evaluation of the  $MDK_{99}$ , which takes into account the inherent probabilistic nature of our measurements and yields a result of  $3.2 \pm 0.7$ , in the case of Fig. 4 a. This approach has three phases: assuming a parametric form to model the function  $S(C, t; \alpha)$ ; extracting maximum likelihood estimates  $\alpha_0$  for the parameters from the empirical data; and, finally, calculating the MDK from the function  $S(C, t; \alpha)$ . The common choice for  $S(C, t)$  is the Zhi model (19)—a time-exponential distribution, where the dependence of the killing rate on concentration is usually taken to be a (decreasing) Hill function (18). To calculate the likelihood, one needs to express the probability of observing growth in a well in terms of the survival function, because the independent trials in this case are the individual wells. Assuming each bacterium's fate is independent of the others in the well, the probability of at least one survivor in a well can be readily expressed. The survival probability is denoted  $S(C, t)$ , so the probability of at least one survivor,  $P(C, t)$ , is 1 minus that of all  $N$  bacteria in the well dying, as follows:

$$P(C, t) = 1 - (1 - S(C, t))^N. \quad (1)$$

Using Eq. 1 as a model function for the observations, the log-likelihood is a sum of log-likelihoods in individual wells, and can be fitted to the data. It is important to emphasize that this approach is not restricted to a large population size, because it models the probability of regrowth, rather than the surviving population size. The effect of Poisson fluctuations in the number of bacteria per well is discussed in Appendix B in the [Supporting Material](#).

### Measurement of the $MDK_{99}$ in tolerant strains

To demonstrate that the MDK measurement can discriminate between different tolerance levels, we implemented the above protocol in a robotic setup and compared the



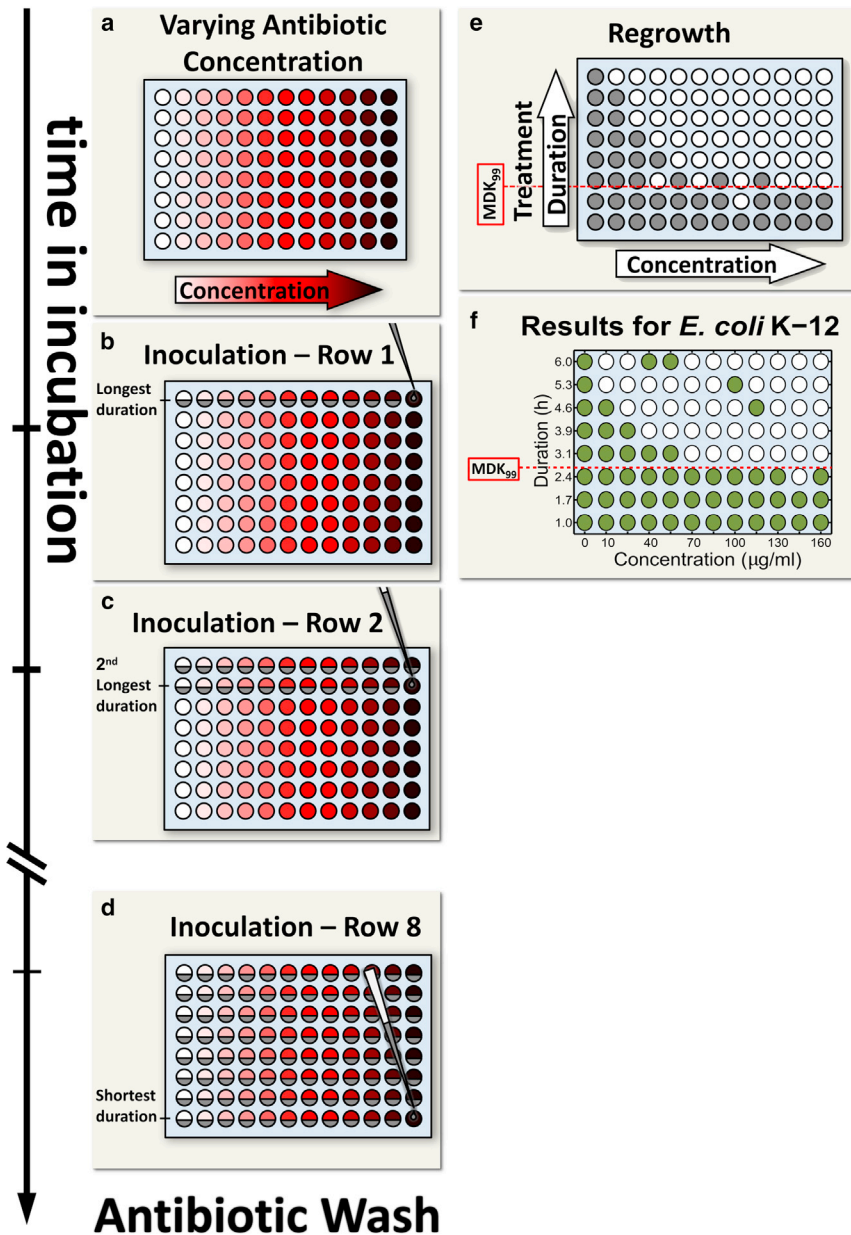


FIGURE 3 The MDK protocol. (a) Shown here is a 96-well plate filled with varying antibiotic concentration. (b–d) Each row of wells is inoculated with 100 CFU per well at different times. (e) Spin-down is performed to wash away antibiotic remains, while retaining the bacteria. After overnight incubation, full wells indicate at least one survivor, and empty wells indicate eradication. The plateau formed by the wells exhibiting visible growth indicates the MDK. (f) Shown here are experimental results for *E. coli* K-12 (KLY) under ampicillin, in agreement with the value extracted from Fig. 1. To see this figure in color, go online.

$MDK_{99}$  of a tolerant mutant (Fig. 4 b) isolated in an evolution experiment under ampicillin treatment (12) to the  $MDK_{99}$  of the ancestral strain (Fig. 4 a). Both strains have identical MIC (12). The comparison between the two MDKs is clear—the  $MDK_{99}$  of the ancestral strain is  $3.2 \pm 0.7$  h, whereas that of the evolved tolerant strain (*tbl5a*) is  $20 \pm 2$  h.

As a test of reproducibility, the MDKs were measured at least 10 times in independent, single-plate experiments (see Table 1).

We conclude that the automated measurement of the  $MDK_{99}$  provides valuable quantitative information concerning the tolerance level of strains, without the need to conduct labor-intensive kill curves.

### Measurement of the MDK in persistent strains

Persistence that is due to a subpopulation of tolerant bacteria, namely a subpopulation that is killed more slowly than the majority population, may also be evaluated with the MDK measurement. If the strain is a high persistence strain with a subpopulation of persister cells  $>1\%$ , the  $MDK_{99}$  measurements will detect the slow killing. Therefore, the  $MDK_{99}$  will enable detection of tolerance of such strains, as shown in Fig. 5 b, where the measurement of  $MDK_{99}$  of the *hipA7* (22) high persistence mutant (MGHY) is shown. On the other hand, a strain containing  $<0.01\%$  persisters, will have a  $MDK_{99}$  identical to that of a nonpersistent strain, and persisters may be detected only by measuring higher MDKs, such as  $MDK_{99.9}$  or  $MDK_{99.99}$

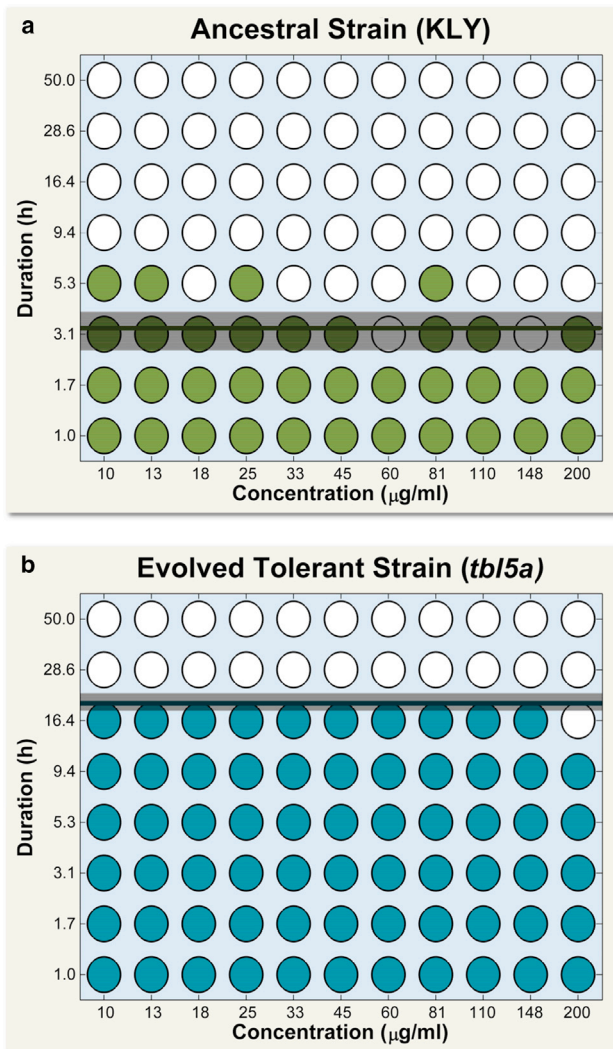


FIGURE 4 Given here is the comparative MDK measurement under ampicillin, using the protocol in Fig. 3. A sensitive ancestral strain (KLY) (a) is compared to a tolerant strain (*tb15a*) evolved from this strain under intermittent antibiotic exposure (12) (b). The lines indicate the  $MDK_{99}$  calculated from these plates alone, and the shadowed area indicates the 95% confidence interval calculated by resampling within each plate (see Appendix B in the Supporting Material). Note that the concentration dependence is not apparent in these data, due to the low time resolution. To see this figure in color, go online.

(i.e., duration for killing 99.9% or 99.99%, respectively). For example, the wild-type *Escherichia coli* has a typical persistence level of  $10^{-3}$  and this low level of persistence is not apparent in its  $MDK_{99}$  ( $2.1 \pm 0.2$  h). However, the measurement of  $MDK_{99.9}$  reveals a longer timescale. From simple arguments (see Appendix B in the Supporting Material), it can be seen that in the absence of persistence, the ratio between  $MDK_{99.9}$  and  $MDK_{99}$ , should be  $\sim 3/2$ . Therefore, the  $MDK_{99.9}$  of wild-type *E. coli* would be  $2.1 \times (3/2) \approx 3$  h. Fig. 5 d compares the expected  $MDK_{99.9}$  of wild-type *E. coli*, based on the  $MDK_{99}$  (Fig. 5 a), to the  $MDK_{99.9}$  measured for this strain

(Fig. 5 c), revealing the biphasic time-kill curve typical of persistence.

We note that our measurements can also provide another way to detect persistence: in the area of the plate in which no growth is supposed to occur (higher  $(C,t)$  pairs), growth will appear in many wells, depending on the persister fraction, reflecting the stochastic occurrence of persisters in each well (see Appendix B in the Supporting Material).

## DISCUSSION

The framework suggested above, in which the MDK is a metric for tolerance, bears several advantages over previous methods. Its definition as a duration is natural, as tolerance is the ability to survive transient exposure to stress. Therefore, the MDK has an inherent advantage over minimum bactericidal concentration/MIC ratios (23), which have been shown to be poorly correlated with tolerance (6,24–26) (minimum bactericidal concentration—the concentration required to kill  $\geq 99.9\%$  of a culture, typically after 24 h incubation). Another option typically used to evaluate tolerance is time-kill curve analysis. However, quantitative parameters that can be compared between experiments are rarely extracted from time-kill curves. Another major disadvantage, apart from the labor-intensive protocol, is that a time-kill curve is often measured at a single concentration. This is problematic because a decrease in lethality of an antibiotic, possibly due to increased MIC, might be misinterpreted as tolerance. Here, we present an alternative automated protocol for easily measuring the  $MDK_{99}$  of a strain without the labor-intensive time-kill curve.

It is advisable to take a number of practical considerations into account when using the MDK protocol. First and foremost, MDK is sensitive to growth conditions, just as MIC and time-kill curves are, because the lethality in most antibiotics is strongly dependent upon growth rate, which, in turn, is directly influenced by the environment and metabolic state (27). A change in medium temperature, or its composition, may cause a significant increase in MDK.

Obviously, the MDK is sensitive to the precise initial inoculum size; however, as the dependence is approximately logarithmic, in cases where there is a slight deviation from a mean of 100 bacteria per well, a simple extrapolation may be used to obtain the desired MDK from the measured results (see Appendix B in the Supporting Material).

It is important to note that the correct choice of treatment duration and concentration ranges is significant. For maximal accuracy of measurement, the concentration range should include sufficiently high concentrations to observe a plateau of killing—a range of concentrations in which the killing rate is constant. The treatment durations should be long enough, so that for most concentrations used there will be both growth and no-growth.

One more problem that occasionally needs to be addressed, especially in long measurements, is the effect of

**TABLE 1 Bacterial Strains**

Strain	Construction	Relevant Phenotype	MDK <sub>99</sub> in Ampicillin <sup>a</sup>	Strain Reference
KLY	P1 transduction of the YFP-Cam cassette from MRR into KL16.	wild-type—low tolerance	2.4 ± 0.4 h	(12)
<i>tbl3a</i>	Tolerant strain was obtained by cyclic exposure of KLY to ampicillin, as described in Fridman et al. (12)	increased tolerance	16 ± 1.5 h	(12)
<i>tbl5a</i>	Tolerant strain was obtained by cyclic exposure of KLY to ampicillin, as described in Fridman et al. (12)	increased tolerance	23 ± 3 h	(12)
EPEC	Wild-type enteropathogenic <i>E. coli</i> , E2348/69 (O127:H6).	wild-type—low tolerance	2.5 ± 0.5 h	(29)
MGY	P1 transduction of the YFP-Cam cassette from MRR lysate to MG1655 (SEQ) from <i>E. coli</i> stock center.	wild-type—low tolerance, low persistence	2.6 ± 0.5 h	(30)
MGHY	P1 transduction of <i>hipA7-tet</i> module from HM22 lysate to MGY.	high persistence	19 ± 2 h	(30)

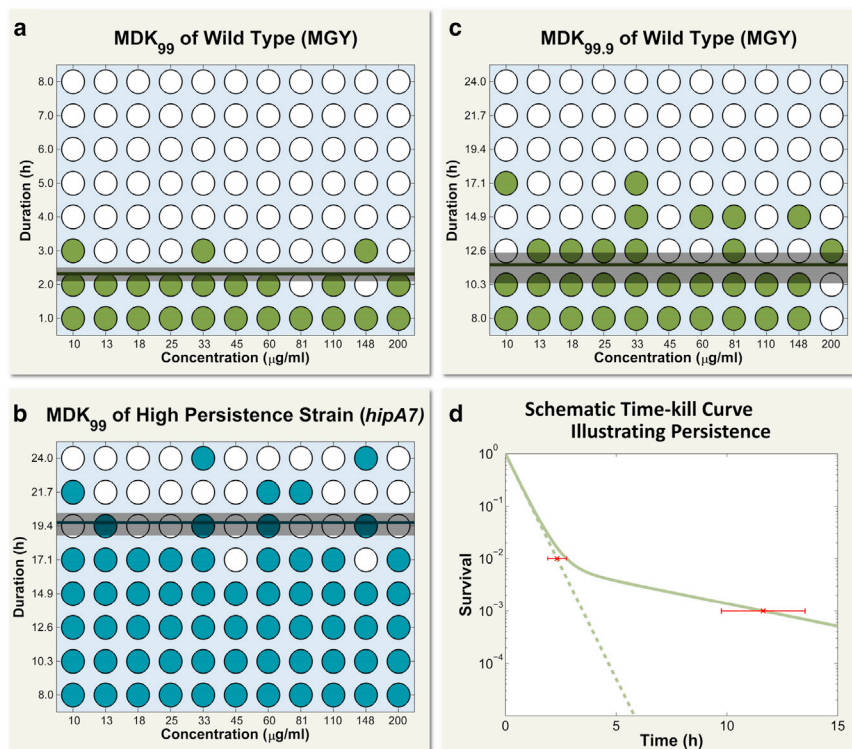
<sup>a</sup>Error margins indicated are standard deviations of 3–10 independent repeats.

extended periods of waiting time on the conditions of the measurement. This may cause a change in the metabolic state of the bacteria awaiting inoculation and affect the results (even though they are on ice; see [Materials and Methods](#)). We ascertained by testing that the waiting time has no significant influence on the MDK<sub>99</sub> results in our strains (see Appendix A in the [Supporting Material](#)). For strains that may be influenced by the duration on ice, the protocol can be adapted to sample bacteria directly from a culture in physiological conditions. This would require the robotic setup to maintain such a culture in constant conditions over time. Other possible pitfalls, common to time-kill experiments, are the significant degradation of antibiotic before inoculation, which can reduce the antibiotic's effectiveness, or evaporation of the medium, which may increase the effective antibiotic concentration. We note, however,

that the MDK test is not sensitive to the precise antibiotic concentration, provided it is high enough to reach saturation. These effects can be corrected for, simply by adjusting the concentration of antibiotic used for the different time points.

## CONCLUSION

With these caveats, the MDK provides a fairly simple and robust measure of tolerance to antimicrobials, and provides a timescale to the problem of combating infection or contamination. We have tested this protocol on several strains, under several antibiotics, and have obtained consistent results (see Appendix C in the [Supporting Material](#)). We hope that the clear definition and probabilistic measurement protocol that we suggest will aid the comparison of results



**FIGURE 5** Measuring persistence using MDK. (a) Given here is the MDK<sub>99</sub> measurement of wild-type *E. coli* (MGY). (b) MDK<sub>99</sub> measurement of high persistence *hipA7* mutant (MGHY) under ampicillin detects high tolerance. High persistence is also apparent in the high noise level. (c) MDK<sub>99,9</sub> measurement reveals the presence of persisters, even in the wild-type strain, when compared to the MDK<sub>99</sub> (a). (d) Given here is the expected schematic kill curve from a strain without persisters and with the same MDK<sub>99</sub> as the wild-type (dotted curve). Note that the MDK<sub>99,9</sub> is expected to be of the order of  $1.5 \times MDK_{99}$ . The actual MDK<sub>99,9</sub> is found to be significantly longer and reflects the bimodal time-kill curve (solid curve). To see this figure in color, go online.



between different labs for a better understanding of tolerance, and will enable more accurate classification and treatment of bacterial infections.

## SUPPORTING MATERIAL

Supporting Materials and Methods and two figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(17\)30551-9](http://www.biophysj.org/biophysj/supplemental/S0006-3495(17)30551-9).

## AUTHOR CONTRIBUTIONS

A.B. performed research, analyzed data, and wrote the article. N.S. contributed ideas and analytic tools. O.F. designed research. N.Q.B. designed research and contributed analytic tools.

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## SUPPORTING CITATIONS

Reference (28) appears in the [Supporting Material](#).

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