



The Physiological State of Bacteria and the Efficacy of Antibiotics

An Experimental and Modeling Odyssey

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Background

Clinical significance: Even in the absence of inherited resistance, antibiotics can fail to clear bacterial infections. A major reason for this is that the infecting population of bacteria includes subpopulations that for physiological reasons are refractory to antibiotics. Perhaps the best (i.e. clinically and epidemiologically most significant) example of this is the latency observed in *Mycobacterium tuberculosis* infections (see P. Ankomah's poster).

Pharmacological PK/PD relevance: The "rational" design of antibiotic treatment protocols focuses on two elements, (i) temporal changes in the effective concentration of the antibiotic in host, pharmacokinetics (PK), and (ii) the relationship between the concentration of the antibiotic and the rate of growth or death of the target bacteria, pharmacodynamics (PD). Although different PK functions are considered, for the most part the PD element of this enterprise focuses on a single parameter, the minimum concentration of the antibiotic required to prevent the growth of the target bacteria, the minimum inhibitory concentration (MIC). MICs, which are also used as a criteria for non-susceptibility, are by official sanction¹, estimated under conditions that are optimal for the antibiotic; low densities (< 10⁶ cells per ml) of exponentially growing bacteria in rich medium and ideal conditions for growth. Needless to say, this hardly reflects a natural clinical setting where bacterial density in infected patients can considerably exceed 10⁹ and may include subpopulations in different physiological states and growing at differing rates.

Current consideration: In an earlier study, we explored mathematically and experimentally, the effects of cell density on the pharmacodynamics of *Staphylococcus aureus* challenged with several antibiotics². In that study the bacteria were growing exponentially at their maximal or near-maximal rate. Here I describe my current experimental and theoretical (mathematical modeling) attempts to extend this consideration of the pharmacodynamics of *S. aureus* populations that are growing at lower rates or are at stationary phase.

Experimental design

All experiments were carried out on the clinical isolate *Staphylococcus aureus* PS80 at 37 °C, in cation-adjusted Muller Hinton broth supplemented with 50 µg ml⁻¹ CaCl₂. Chemostats were of the design described in Chao *et al* (1977)³. Population density was estimated by counting colony forming units (cfu) growing on Luria Bertani agar.

Results

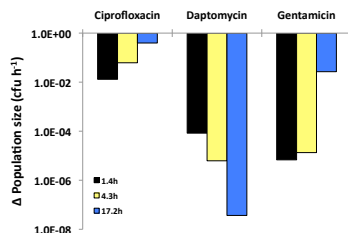
Growth rate affects antibiotic killing non-uniformly

One would anticipate that as the rate of cell division declines, antibiotics would be less effective due to the reduced metabolic rates of the target bacteria.

Using chemostat cultures set at three different dilution rates, we challenged these exponentially growing cells to the three most effective "bactericidal" antibiotics from our earlier study, ciprofloxacin, daptomycin and gentamicin. Contrary to expectation, the rate of kill of these antibiotics does not necessarily decline monotonically with the rate of cell-division.

- I. For ciprofloxacin, there is a seemingly direct relationship between growth rate and killing efficacy.
- II. For gentamicin, killing efficacy appears to be the same at both fast and moderate growth rates (1.4h and 4.3h doubling times, DT), but is much reduced when the population is dividing at a low rate (17.2h DT).
- III. For the much touted daptomycin (a relatively new antibiotic) there is a peculiar inverse relationship between killing efficacy and the pre-exposure growth-rate.

Figure 1. Change in population size (per h) of cells growing in chemostats at different dilution rates and exposed to 100 MIC ciprofloxacin, daptomycin and gentamicin. Samples were taken at 15 minute intervals for the first hour and every half hour for X hours. The plotted change in population size was calculated from the maximum slope of CFU estimates of viable cell densities as a function of time.



Ageing stationary phase cultures become increasingly refractory to antibiotics

One may expect that older cultures to be increasingly resilient and thus more refractory to antibiotics or alternatively the opposite due to accumulation of toxic products in older cells. To ascertain the effects of term in stationary phase on the killing efficacy of antibiotics, I added antibiotics to variably "aged" stationary phase cultures of *S. aureus* PS80 (growth for 1, 3, or 7 days). To control for the pH increase observed in such medium (sans glucose), I adjusted the pH of these cultures to pH 7 prior to the introduction of the antibiotics (buffered growth medium impairs antibiotic function). Compared to the antibiotic-free control, cell densities declined to some extent in all samples.

- I. There is variation in the extent and rate at which these antibiotics kill.
- II. The term in stationary phase adversely effects antibiotic; the 3d and 7d cultures were substantially more refractory than the 1d culture. This was most evident in the 3 hour samples which is suggestive of the effect being on the rate of killing.
- III. The distinction between bactericidal and bacteriostatic antibiotics is a misnomer as linezolid (LIN) and vancomycin(VAN) are believed to be "bacteriostatic".
- IV. Oxacillin (OXA), the frontline drug for treating *S. aureus* infections, isn't as impressive as gentamicin or daptomycin

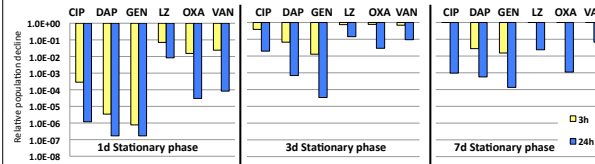


Figure 2. Effect of culture age on antibiotic efficacy. Batch culture grown cells of different ages were challenged with 100x MIC of each antibiotic excluding ciprofloxacin and linezolid (20x MIC). Samples were taken in triplicate at 3h and 24h to estimate the survivor population density by plating on LB agar plates. We report the decline in population density relative to the antibiotic-free control at each timepoint (CIP=ciprofloxacin, DAP=daptomycin, GEN=gentamicin, LZ=linezolid, OXA=oxacillin, VAN=vancomycin.).

Aged media induce refractory state in exponentially growing cells

The spent medium from stationary phase cultures of all 3 ages was more than sufficient to support the growth of low densities of cells. The extent of residual growth declines with the age of the culture, suggesting active metabolism even at stationary phase. To ascertain whether the observed decline in killing efficacy in the above experiment is a response to the medium, low densities of exponentially growing cells introduced into the spent medium and challenged with these antibiotics.

- I. Relative to fresh media (F) the extent of killing of erstwhile exponentially growing cells declined with the "age" of the media.

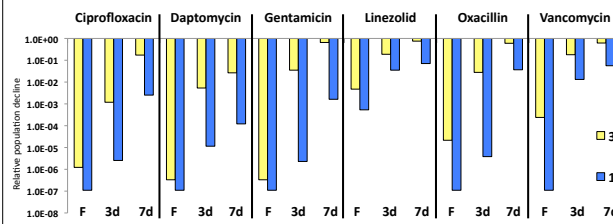


Figure 3. Observed decline in the population size of an exponentially growing culture of *Staphylococcus aureus* ps80 after 3h (3) and 18h (18) of antibiotic-challenge in either fresh (F), 3 day spent (3d), or 7 day spent (7d) media. The experiment was carried out under batch-culture conditions at 37 °C.

Modeling Growth-rate and Density-dependent PD

In an effort to better understand the dynamics of antibiotic treatment and to generate testable hypotheses to explore the effects of bacterial physiology on the PD of antibiotics and antibiotic treatment, I extended our previous PD model⁴ to allow for changes in the physiological state of the bacteria. In this model, the rate of growth (or death) of the bacteria is a hill function that depends on the bacteria's density, D, (cfu/ml) the antibiotic concentration, A, (µg/ml), and as a surrogate for physiological state, the concentration of some limiting resource, R, (µg/ml).

$$\theta(A, D, R) = \Psi_{Max}(R) \cdot \left[\frac{(\Psi_{Max}(R) - \Psi_{Min}(R)) \cdot \left(\frac{A}{M(D)} \right)^c}{\left(\frac{A}{M(D)} \right)^c + (\Psi_{Min}(R) / \Psi_{Max}(R))} \right]$$

I assume that the maximum and minimum realized growth rates are monotonic increasing functions of R, and for the latter (Ψ_{min}), we introduce a constant, c (0 < c < 1) which determines the extent to which antibiotic-mediated killing is affected by resource [R].

$$\Psi_{max}(R) = V_{max} \frac{R}{(R + km)}$$

$$\Psi_{min}(R) = V_{min} + cV_{min} \frac{R}{(R + km)}$$

$$M(D) = M_{min} + pd \left(\frac{M_{max}}{D + K_A} \right)$$

V_{max} and V_{min} represent the medium-specific maximum and minimum rates of growth and km is the resource concentration where the growth or death rate is half its maximum value. As shown as well as assumed in Udekwo *et al* (2009), the MIC increases with density as a logistic model where pd accounts for the extent of MIC increase with population density, M_{min} represents the minimum or basal MIC, M_{max} is the maximum MIC, K_A is the density at which the density effect is half its maximal value.

Experimental testing of model-generated predictions

Prediction 1- As the stationary phase population is killed, the cell density declines and increasing amounts of resources will be made available as the dead cells are converted into nutrients (not dust). As a result, the killing efficacy of the antibiotics should increase with time (see Figure 4).

- My preliminary results suggest that this is more likely in the cases where the antibiotic's Ψ_{min} parameter is closer to 0 (slower killers) than for those with more negative Ψ_{min} term

Prediction 2 The stationary phase population may include cells that are turning over and being killed and those that are not and are refractory to the antibiotic (persistence⁵) that increase in relative frequency as time proceeds (a situation also covered in our model). If this is the case, the dynamics of the killing process would be different than predicted in the absence of such a population. The extent of killing would level off rather than continue until all the bacteria are done-in.

- Preliminary work carried out on *S.aureus* has shown there to be induction of a 'persister'-like physiological state during antibiotic exposure. It is as yet unclear to what extent this does or does not mimic the dynamics of antibiotic-mediated killing of stationary phase populations.

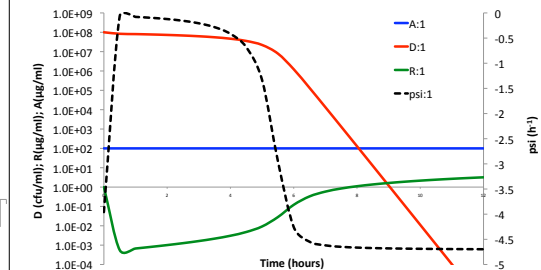


Figure 4. a) Change in predicted growth or death rate Ψ (black), resource concentration [R] (green), in one representative run of our model with start density 1×10^8 cfu/ml, initial resource concentration was 1µg/ml and with a moderate density effect on the MIC. Predicted population density is shown in red and we assume a constant antibiotic concentration. Parameter values: $pd = 0.5$; $V_{max} = 1$; $V_{min} = -5$; $M_{min} = 1$; $M_{max} = 10$ and $K_A = 1 \times 10^9$ cfu/ml

Currently, I am in the process of experimentally determining the values of the parameters for each bug : drug combination and will happily provide more information on the model as well as the experimental work.

References

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