Ecology of antibiograms: when drug diffusion accelerates the evolution of resistance

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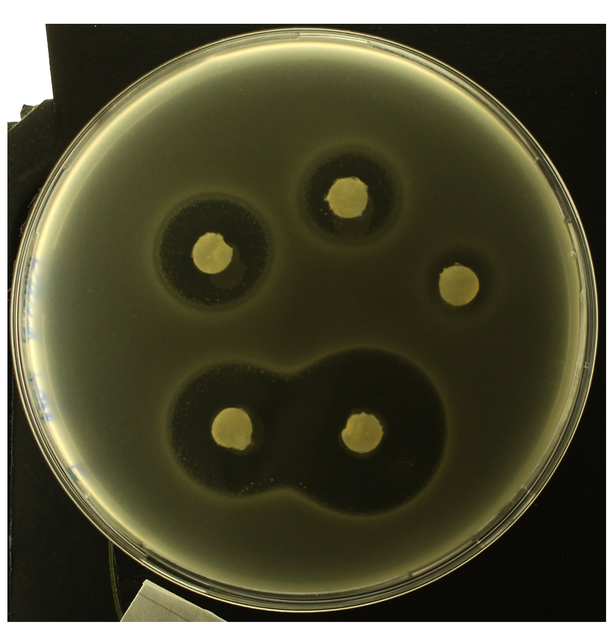
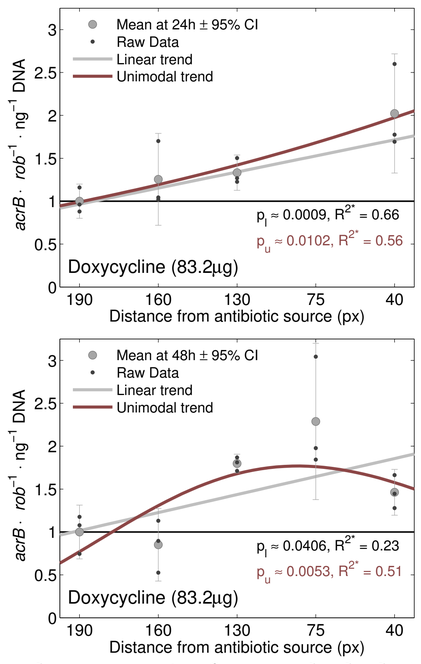
FINDINGS





Bullseye patterns are commonly found in antibiograms, yet why they appear is often overlooked. Antibiotic gradients, like those generated during an antibiogram (above), force conditions that promote the rapid onset of drug resistance. By inhibiting bacterial growth, antibiotics protect the surrounding carbon (nutrients) and therefore only resistant mutants will be able to use it generating bullseye patterns in the process. We detected that mutants with additional copies of the operon *acr*, responsible for the AcrAB-TolC efflux pump, emerged within 24h and spreaded as predicted by Fisher's traveling wave. This amplification of *acr* is consistent with data observed in the clinic[1].

I. OBJECTIVE

ANTIBIOGRAMS are one of the most common antibiotic sensitivity tests used in hospitals, but they also provide a unique tool to study the ecology of antibiotic resistance. Antibiograms, like the one shown above, produce a clearance or inhibition zone due to the effect of the drug and it can be used to translate antibiograms into a dose regime. But this translation is problematic[2].

We developed a mathematical antibiogam to study the diffusion of antibiotic molecules in these tests and resolved the relationship between zone of inhibition size and drug dose that we validated *in vitro*. We then used the solution to modify Monod's growth law and study the growth of microbes in the presence of antibiotic gradients. We observed the onset of resistance to be very rapid (8h), leading to emerging bullseye patterns in which bacterial subpopulations are spatially arranged based on their degree of resistance. We found each subpopulation to contain increasing number of the *acr* operon, known to confer drug resistance in the clinic[1].

II. METHODS AND RESULTS

II.A Linear diffusion theory describes how drug molecules diffuse

Medicine and general education textbooks[2–6] claim that zone of inhibition size is proportional to drug concentration. Given this proportionality, an antibiogram can be translated into a dose of reference, like the minimum inhibitory concentration (MIC), using linear regressions[6,7]. However, it is difficult to demonstrate that the data is truly straight[6,7] and, importantly, the aforementioned claim confronts fundamental linear diffusion theory.

Using the linear diffusion equation $A_t = \sigma(A_{xx} + A_{yy})$, which describes the flux of the antibiotic A in two dimensions x and y, we derived the following relationship between the radius of the zone of inhibition and drug concentration: $r = \sqrt[2]{A_c/A_d \pi e}$, where A_c denotes the initial drug concentration, A_d the minimum inhibitory concentration and $r^2 = x^2 + y^2$ the radius of the zone of inhibition (Fig. 1A).

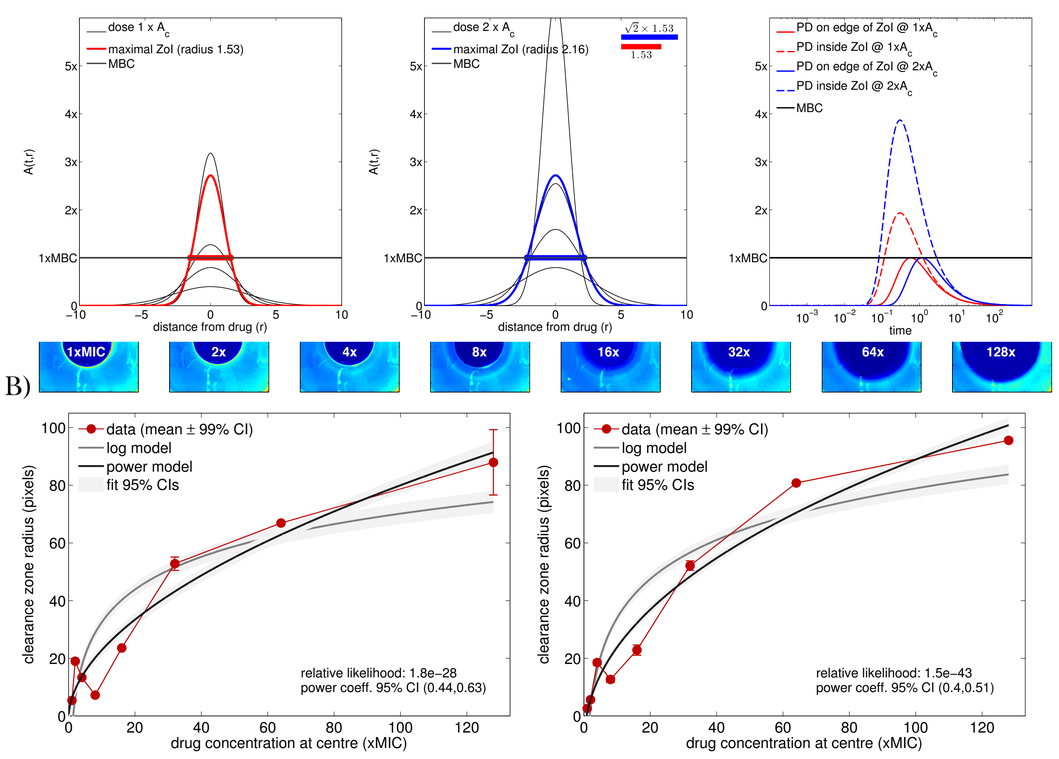


FIGURE 1. Antibiotic molecules diffuse as predicted by linear diffusion theory. A) The mathematical antibiogram predicts that doubling drug concentration will not double the size of the zone of inhibition but by a factor of $\sqrt[2]{2}$. B) Antibiograms implemented to force drug diffusion in two dimensions. Bacterial lawn (in false colour) showing the zones of inhibition with dosages ranging from 1 to 128 times the MIC for doxycycline. C) Data obtained for *Escherichia coli* MG1655 (left) and AG100 (right) is non-linear and consistent with a power model with coefficient two (reminder: $\sqrt[2]{a} = a^{0.5}$).

We implemented an antibiogram protocol where the drug diffuses strictly in two dimensions (Fig. 1B) and registered the growth over time using a computerised camera. The resulting data demonstrates that the increase in zone of inhibition is consistent with the expression above derived from linear diffusion theory (Fig. 1C) and, consequently, to double the zone of inhibition the antibiotic dose must increase 4-fold (8-fold if diffusion is in 3D).

II.B Antibiotic gradients lead to the rapid (8h) onset of resistance

Antibiotic resistance is studied in environments that assume uniform drug distribution[8,9] but this assumption is not realistic as gradients are everywhere in nature. Now that we demonstrated how drug molecules diffuse, we modified Monod's growth law to accomodate the spatial distribution of a source of carbon, S, and an antibiotic A. Assuming that S is uniformly distributed at t=0, the spatially-extended Monod model predictics the maximal growth rate to occur not at the edge of the plate where the drug concentration is minimal, but somewhere closer to the source of antibiotic. We found this to be true in the data (see Fig.3A and B, below).

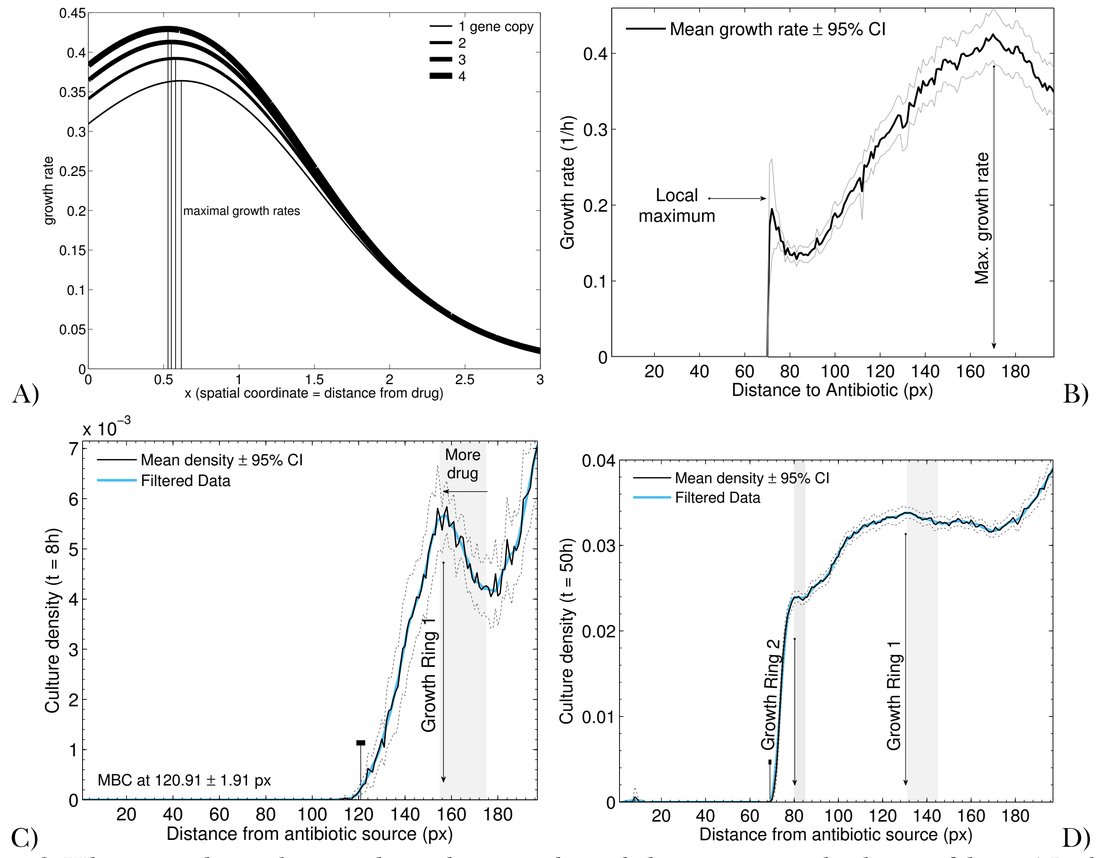


FIGURE 2. When exposed to antibiotic gradients, the maximal growth does not occur in the absence of drug. A) Prediction of how bacterial growth rate changes as a function to the distance to the antibiotic, assuming 1-4 copies of a gene that confers resistance to the drug. B) Empirical relationship between growth rate and distance to antibiotic based on the computationally-recreated growth curves. C) Dose-response profile observed after an incubation of 8h. Note that this profile is non-monotone. D) Analogous dose-response observed after 50h. The presence/absence of growth rings was calculated based on the winding number of the dose-response profile.

This results in non-monotone dose-response profiles, where higher drug concentrations leads to higher cell density, after just 8h of exposure to the drug. In a spatial context these profiles define rings of growth and, in our experimental conditions, we managed to detect two rings that appear at different incubation times (Fig. 3C and D).

II.C Spread of AcrAB-TolC mutations consistent with Fisher's travelling wave

We then focused on the multidrug efflux pump AcrAB-TolC and used a strain of *E. coli* where this pump is tagged with green fluorescence protein (GFP). Based on photographic data, we recreated the relative abundance of this pump as a function of time and distance to antibiotic source as the ratio between cell density using non-filtered and GFP filtered light. This ratio was two to three-fold that observed at the edge of the plate where the concentration of antibiotic is minimal (Fig. 3, left).

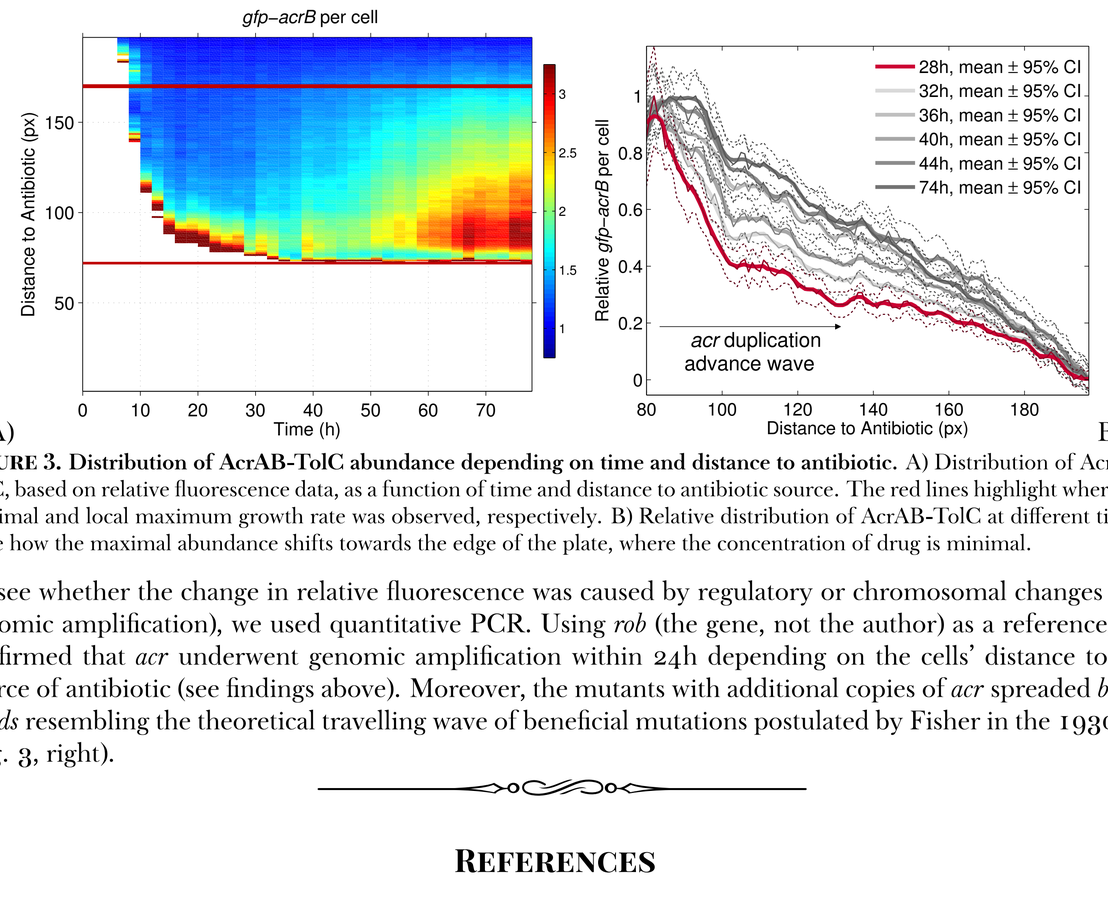


FIGURE 3. Distribution of AcrAB-TolC abundance depending on time and distance to antibiotic. A) Distribution of AcrAB-TolC, based on relative fluorescence data, as a function of time and distance to antibiotic source. The red lines highlight where the maximal and local maximum growth rate was observed, respectively. B) Relative distribution of AcrAB-TolC at different times. Note how the maximal abundance shifts towards the edge of the plate, where the concentration of drug is minimal.

To see whether the change in relative fluorescence was caused by regulatory or chromosomal changes (i.e. genomic amplification), we used quantitative PCR. Using *rob* (the gene, not the author) as a reference, we confirmed that *acr* underwent genomic amplification within 24h depending on the cells' distance to the source of antibiotic (see findings above). Moreover, the mutants with additional copies of *acr* spreaded *backwards* resembling the theoretical travelling wave of beneficial mutations postulated by Fisher in the 1930s[10] (Fig. 3, right).

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