Evolution of Drug Resistance in Bacteria

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Abstract Resistance to antibiotics is an important and timely problem of contemporary medicine. Rapid evolution of resistant bacteria calls for new preventive measures to slow down this process, and a longer-term progress cannot be achieved without a good understanding of the mechanisms through which drug resistance is acquired and spreads in microbial populations. Here we discuss recent experimental and theoretical advances in our knowledge how the dynamics of microbial population affects the evolution of antibiotic resistance. We focus on the role of spatial and temporal drug gradients and show that in certain situations bacteria can evolve *de novo* resistance within hours. We identify factors that lead to such rapid onset of resistance and discuss their relevance for bacterial infections.

Key words: antibiotic resistance, modelling infection, biological evolution

1 Introduction

Since their inception in the beginning of the 20th century, antibiotics have been an indispensable tool used to treat and prevent bacterial infections and, together with other health care improvements, have revolutionized medicine. However, penicillin-resistant bacteria emerged in just a few years after the introduction of penicillin [21], and it is now widely acknowledged that resistance to antibiotics is becoming a global thread [16]. Different counter-measures have been proposed [52, 1, 2], including reducing the use of antibiotics, better surveillance methods or incentives for the pharmaceutical industry to develop new antibiotics. However, the battle against antimicrobial resistance

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(AMR) can not be won unless we fully understand the molecular basis of resistance, how it spreads among microorganisms, and how it is selected for.

This chapter reviews theoretical and experimental progress in our understanding of the evolution of AMR. We focus on the role of population dynamics and spatial and temporal heterogeneities - the areas which have strongly benefited from a physics-like approach. We briefly discuss relevant experiments and show how their outcomes can be understood using simple mathematical models. In the last section we also speculate on the significance of *in vitro* experiments and theoretical models to bacterial infections *in vivo*.

We do not discuss molecular mechanisms behind AMR [7, 18] except where necessary. This gives our discussion a more general flavour. In fact, many analogies exist between AMR and resistance to cancer chemotherapy [36], and theoretical models similar to discussed in this chapter have been applied to cancerous tumours [22, 23] and viral infections [46].

2 Quantifying resistance

Drug resistance refers to the ability of a pathogen to grow in the presence of a drug. Many different measures have been proposed to quantify resistance. One of them is the minimal inhibitory concentration (MIC), above which no growth can be detected in a standardized experimental protocol [4]. Different bacteria and antibiotics have very different MICs [4]. MIC can be as low as $\sim$ 10ng/ml (fluoroquinolones, susceptible bacteria) and as high as $\sim$ 10mg/ml (beta-lactams, beta-lactamase carrying bacteria) depending on what enzyme the antibiotic targets, the permeability of the cell membrane, and other factors. Another popular way of quantifying resistance is IC_{50} - the concentration of the drug at which the growth rate decreases by 50% in the exponential phase of growth. A yet more complete characterization of the response to an antibiotic can be obtained by measuring the growth rate at different drug concentrations [55, 26]. These "kill curves" are non-universal and depend on the antibiotic, bacterial strain, and growth conditions [26], but they often follow a sigmoidal shape (Fig. 1, left).

High levels of resistance are typically mediated by genes that code enzymes degrading or pumping out the drug [21]. Such genes are often acquired from other microbial species in the process of gene exchange. However, low-to-moderate resistance can also evolve *de novo*, sometimes following only a brief exposure to the drug. A single-nucleotide changing mutation that alters the binding site of the antibiotic may be enough to increase the MIC tens of times or more [13, 42, 29], and a combination of a few such mutations may be enough to increase it a few thousand times [42]. Such mutations occur with probabilities 10^{-9} to 10^{-7} per nucleotide per replication [25, 60, 37, 51]. In what follows we shall consider only *de novo* evolution of AMR.

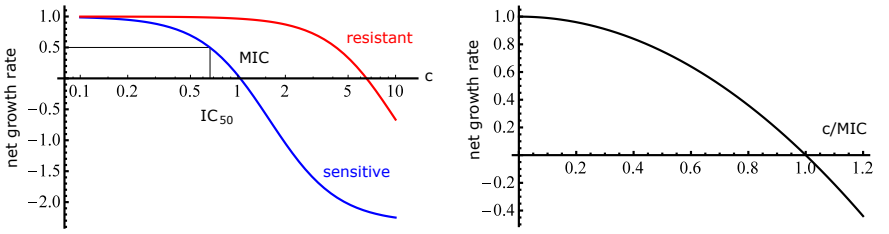

Fig. 1 Left: A typical kill curve (blue) versus antibiotic concentration c. Net growth = replication minus death. IC_{50} and MIC are defined as c at which growth is 50% of the maximal rate and zero, respectively. The net growth can become negative above MIC if the drug kills the cells or the cells are removed by another mechanism. The curve for the resistant strain (red) is shifted to larger c and hence it has higher MIC and IC_{50}. Right: growth curve $g = 1 - (c/\mathrm{MIC})^2$ from Eq. (2) used in mathematical models in this chapter.

3 Evolution of resistance in homogeneous environments

In many laboratory experiments on AMR [28, 53, 33, 41], bacteria grow in a shaken flask, a microtiter plate, or a chemostat. Shaking or stirring mixes the content so that all bacteria experience identical conditions, including the same concentration of the antibiotic. After a few tens of generations bacteria often evolve resistance in these experiments when exposed to sub-lethal concentrations of the drug.

To gain insights into what determines the rate of AMR evolution in such experiments, we shall study a simple mathematical model of a population of N bacteria of different genotypes m with different resistance levels (different MICs). Let $m = 0$ be the most sensitive (least resistant) genotype and let the MIC increases monotonously with m (Fig. 2, top). Genotype m reproduces at rate g_m which increases with m. Reproduction is assumed to be balanced by death so that the size remains approximately constant[1]. Genotype m can mutate into genotypes $m \pm 1$ with probability μ per cell per replication.

We want to calculate the rate u at which a new, more resistant mutant reproducing at a higher rate $g_{m+1} > g_m$ will emerge and take over the population. If the population size is much smaller than the inverse of the mutation rate, the rate-limiting step is the process of generating resistant mutants. The rate u is then the product of the rate $\mu g_m N$ with which the mutants are created, times the probability F that a mutant fixes (becomes the dominant organism) in the population. The fixation probability F can be obtained using the standard result of population genetics [50] and is approximately equal to the selective advantage of mutant $m+1$ over mutant m: $F = (g_{m+1} - g_m)/{g_{m+1}}^2$[2]. The rate u is then $\mu g_m N F$ and the average time to resistance can be estimated as

[1] This can be achieved experimentally by serially diluting the culture when it reaches saturation, or by continuously diluting it in a chemostat.

[2] This is true as long as $F \gg 1/N$.

$$T_{m\to m+1} = 1/u = \frac{g_{m+1}}{\mu N g_m (g_{m+1} - g_m)}. \quad (1)$$

Each of the growth rates $\{g_m\}$ is a function of the antibiotic concentration c. For the sake of simplicity, we shall assume that

$$g_m = g_{\max}\left(1 - (c/\mathrm{MIC}_m)^2\right), \quad (2)$$

so that all g_m look the same when plotted against the "normalized" concentration c/MIC_m (Fig. 1, right). Equation (2) is of course only an approximation to the real kill curves, but for $c < \mathrm{MIC}_m$ it qualitatively reproduces the curves for many antibiotics (c.f. Ref. [55]).

3.1 Time-independent drug concentration

Let us now apply Eq. (1) to the case of fixed concentration c and the initial population consisting of the sensitive strain $m = 0$. If $c > 0$, the antibiotic imposes a selection pressure on the sensitive strain. If the antibiotic concentration is below MIC_0 and the sensitive strain can grow ($g_0 > 0$), a faster-growing mutant $m = 1$ will eventually emerge. After some time $T_{0\to 1}$ the population will consist mainly of genotype $m = 1$, which will then produce mutant $m = 2$ etc. Importantly, however, the time $T_{1\to 2}$ will typically be larger than $T_{0\to 1}$ because the difference $g_2 - g_1$ is smaller than $g_1 - g_0$. To see this more clearly, let us consider a particular, if slightly artificial, example of g_m given by Eq. (2) with $\mathrm{MIC}_m = 2^m$. The MICs of the genotypes $m = 0, 1, 2, 3, \ldots$ in this example are $1, 2, 4, 8, \ldots$ (arbitrary units) and the growth rate decreases quadratically with increasing c. This is our "standard model" that we shall use in this chapter. From (1) we obtain that

$$T_{m\to m+1} = \frac{4^m\left(4^{m+1} - c^2\right)}{g_{\max} 3\mu N c^2\left(4^m - c^2\right)}. \quad (3)$$

Figure 2, left, shows how quickly $T_{m\to m+1}$ increases with m. This is mainly caused by the fixation probability F decreasing fast with m. Intuitively, this is to be expected - each new mutation increases the MIC but does not increase the growth rate as significantly as the previous mutation if drug concentration is kept constant. This means that although evolving a moderately resistant strain requiring only a single mutation ($m = 1$) does not take very long, evolving a strain with multiple mutations $m \gg 1$ is a very slow process because the total time $T_{0\to 1} + T_{1\to 2} + \ldots + T_{m-1\to m}$ increases quickly with the number m of mutational steps.

This is confirmed in a stochastic simulation in Fig. 2, right. We assume that cells die with rate d and replicate with rates $g_m = (1 - (c/2^m)^2)(1 - N/K)$. Here N is the total population size, and K is the carrying capacity. The term

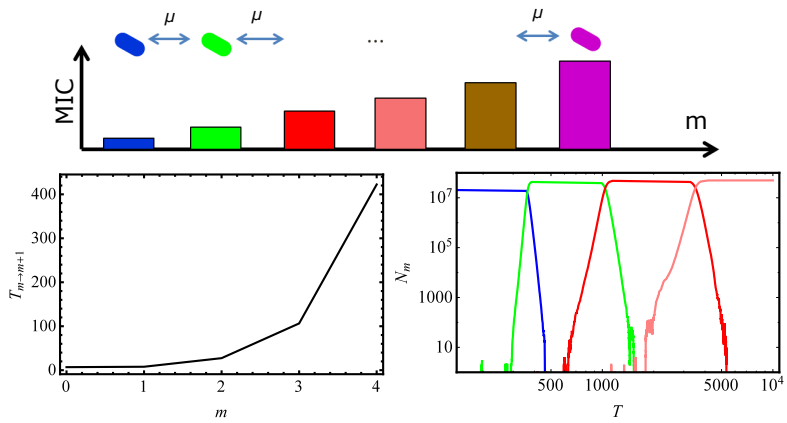

Fig. 2 Top: allowed mutations between genotypes $m = 0, 1, 2, \ldots$. Bottom left: plot of the times $T_{m\to m+1}$ to the next mutant $m + 1$, for $c = 0.9$ and $\mathrm{MIC}_m = 2^m$. Bottom right: a single simulation run for a stochastic model with carrying capacity $K = 10^8$, death rate $d = 0.5$, mutation probability $\mu = 10^{-9}$, and $c = 0.6$. Colours blue, green, red, and pink (as in the top panel) correspond to $m = 0, 1, 2, 3$. We can see consecutive fixation of the green, red and pink mutants which replace the previous genotype, as well as failed fixation attempts ("spikes" with $N_m < 10$). This process will continue until there are no mutations that could further increase the MIC, or the selective advantage F of the new mutant becomes comparable to $1/K$. All further mutations behave as neutral mutations and their fixation is extremely rare (probability $F = 1/K$).

$1 - N/K$ decreases the growth rate as the carrying capacity is approached, limiting the total population size. Fig. 2, right shows that it takes 10x less time to evolve mutant $m = 1$ than to evolve the 4x-more-resistant mutant $m = 3$. Note that Eq. (3) agrees only quantitatively with this result because it uses maximal rather than actual growth rates. When the actual rates are taken into account, the agreement becomes quantitative [27].

3.2 Evolution in time-varying drug concentration

We shall consider two processes in which the concentration $c(t)$ of the antibiotic changes in time:

1) $c(t)$ varies according to a fixed protocol, independent of the actual state of the population. This is the case of a typical antimicrobial therapy in which a patient takes the medicine at regular intervals, regardless of the present size or genetic composition of the microbial population. In an *in vitro* experiment, this amounts to e.g. adding progressively more and more antibiotic to a batch bacterial culture, or gradually increasing the antibiotic influx in a chemostat.

2) $c(t)$ is continuously adjusted to maximize selection while avoiding killing the population. The size of the population, monitored e.g. by measuring the turbidity of the sample, is used in a feedback loop to control the influx of the drug. An example of a device with such a feedback is the morbidostat [62].

Process (1) can lead to a rapid evolution of resistance only if $c(t)$ increases fast enough to be always close to the MIC of the most-resistant genotype in the population, but not exceeding it – otherwise, the population dies out. For a low removal rate d, the critical time $T_{\rm crit}$ over which the concentration increases from $c \ll$MIC to $c \approx$MIC must be larger than the time to produce at least one resistant mutant. In the case of subsequent fixation of beneficial mutants and for small populations ($\mu N \ll 1$), this critical time is

$$T_{\rm crit} \approx 1/(\mu g_m N), \tag{4}$$

whereas in a large populations in which new mutants occur frequently, the rate-limiting step is the growth from one to enough many cells to produce a mutant and hence

$$T_{\rm crit} \approx (1/g)\ln(1/\mu). \tag{5}$$

The rate at which the drug concentration increases must be such that the above equations are fulfilled for all mutants; too slow and the evolution of resistance takes very long, while too fast kills the population. This is hard to achieve in practice unless we know the growth curves, mutation rates, etc. parameters very precisely which is almost never the case. This is illustrated in a computer simulation in Fig. 3, top - a small change in the increase rate of the concentration over time completely changes the fate of the population (rapid evolution of resistance versus extinction).

Process (2) does not require any knowledge of the MICs, except that the initial concentration of the antibiotic must be below the MIC of the sensitive strain. By systematically increasing c, the time to evolve $m+1$ from m can be kept very small (cf. Eq. (3)). Fig. 3 bottom shows a simulated evolution of our standard model in the case when c is increased by 0.5 every hour if the total bacterial count is above 30% of the carrying capacty K, and decreased by the same amount if the bacterial count is below that value. As expected, evolution proceeds very quickly and soon the most resistant mutant takes over. This rapid evolution agrees with what has been experimentally observed in Ref. [62].

4 Evolution of resistance in heterogeneous environments

A uniform distribution of the drug is easy to achieve in the lab but is very unlikely in real infections, where imperfect tissue permeability, drug degra-

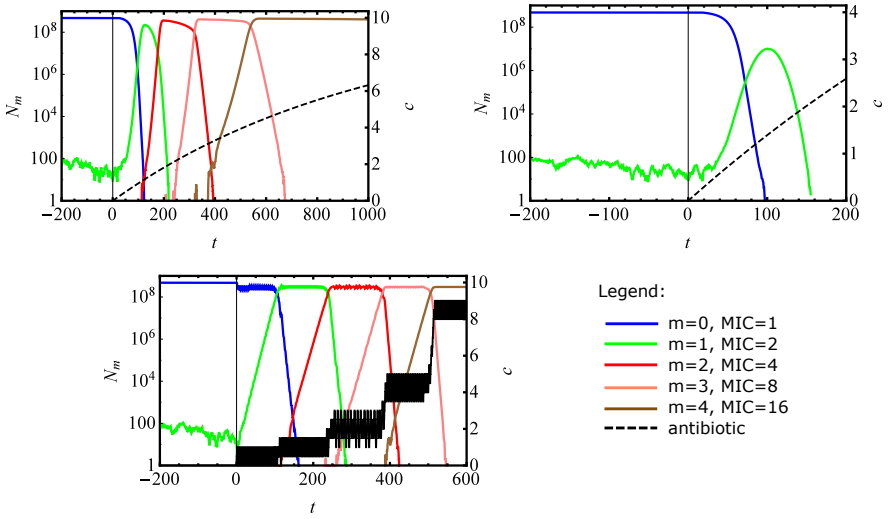

Fig. 3 Top: Examples of simulation runs in which antibiotic concentration increases in a pre-determined way as $c(t) = 10(1 - e^{-\alpha t})$ for $t > 0$ (dashed curves). α is the increase rate of the drug concentration. Such $c(t)$ would be typical for a chemostat experiment with constant influx of the drug for $t > 0$. Left: $\alpha = 0.001$, right: $\alpha = 0.0015$. Different coloured curves correspond to different mutants from Fig. 2(top), see also the legend. A 50% change in the increase rate α causes a switch from fast evolution (left) to no evolution and extinction (right). Bottom: simulated "morbidostat" in which $c(t)$ is adjusted in response to population's growth to exert strong selection pressure at all times. Parameters: $K = 10^9$, the rest as in Sec. 3.1.

dation, binding to proteins etc., cause the concentration to vary in space. The effect of a non-uniform drug distribution was first studied experimentally in a 2x2cm microfluidic "death galaxy" [67, 11], in which a gradient of the antibiotic ciprofloxacin was created by pumping two different growth media (with and without the drug) along the opposite sides of the device. Diffusion and partial mixing created a stable gradient in the centre of the device. Surprisingly, bacteria with MIC more than 200x that of the parental strain emerged in as little as 10h in this experiment, whereas no resistant bacteria were detected in a well-mixed control experiment. Resistance was caused by point mutations in four different genes [67].

4.1 Computer model

Two computer models have been proposed [30, 27] [3] to explain these results. Although slightly different in details, both models assume the same

[3] See also earlier work [32] on HIV infections.

spatial and genetic structure. Bacteria inhabit a series of weakly-connected compartments containing increasing (from left to right) amounts of the antibiotic (Fig. 4, top). Bacteria migrate between the compartments with rate m and replicate if the antibiotic concentration is below their MIC. Each bacterium can increase or decrease its MIC by mutating along a one-dimensional mutational pathway as in our standard model from Fig. 2, top.

A kymograph of a typical simulation run for the model from Ref.[27] is plotted in Fig. 4, bottom. As in the experiments [67], resistance evolves faster and higher MICs are obtained in the presence of an antibiotic gradient than if the antibiotic is uniformly distributed. In the gradient case, the population advances in waves, each corresponding to a new, better adapted mutant. Although the mathematical theory explaining the difference between the two cases involves some algebra, a qualitative understanding can be achieved using a much simpler and intuitive reasoning described below.

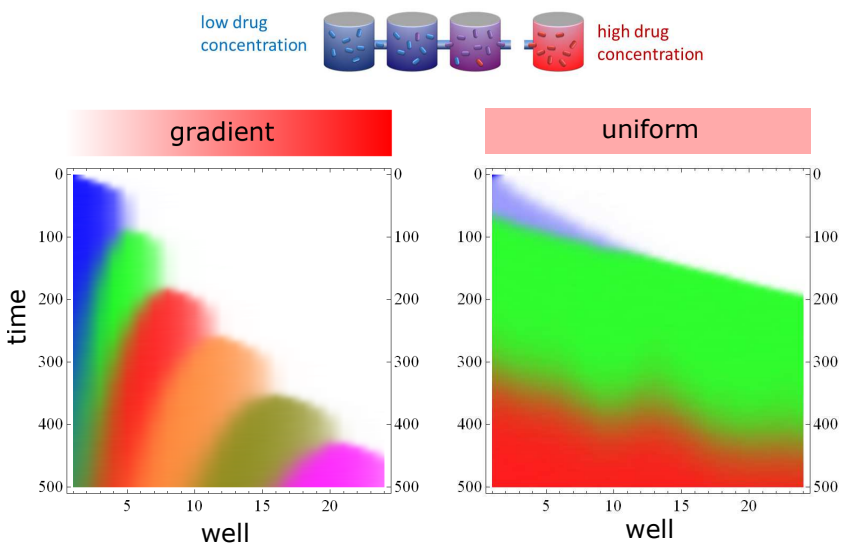

Fig. 4 Top: spatial structure of the model with non-uniform drug distribution. Antibiotic concentration $c(x)$ increases from left to right. Left/right: kymographs of two simulation runs for the uniform $c(x) = 0.9$ and non-uniform $c(x) = \exp(3x/24) - 1$ drug distribution. Horizontal axis represents space (wells), vertical axis represents time. Colours represent different mutants as in Figs. 2, 3. The purple mutant (MIC=32) evolves in the gradient case at $t \approx 450$, whereas only a moderately-resistant red mutant (MIC=4) can be seen at the same time in the uniform case. In each case, $L = 24$ wells, $K = 10^5$ per well, mutation probability $\mu = 10^{-5}$, migration rate (rate at which bacteria jump between the compartments) $m = 0.02$; other parameters are the same as in Sec. 3.1 and Fig. 3.

4.2 Intuitive explanation

It turns out that a system with a spatially non-uniform drug distribution behaves somewhat similar to a homogeneous system in which drug concentration increases in time in a way that maximizes selection pressure (Sec. 3.2). We can understand this using the well-mixed formula (1) applied to the spatial model near the population's edge. Albeit the effective population size N can be quite small as replication is possible only in a narrow region at the edge, this is offset by a large increase in the selective advantage new mutants have at the edge. Let us look at what happens to a population with the same MICs and growth curves as in Sec. 3.1. The population of genotype m will move forward until the antibiotic concentration $c \approx \mathrm{MIC}_m = 2^m$ becomes high enough so that the net growth rate (replication minus death) becomes zero. However, if a mutant $m+1$ occurs near the front where $g_m = \epsilon$ is non-zero but small, its growth rate will be $g_{m+1} \approx (1/2)g_{\max}$ and hence

$$T_{m\to m+1} \approx \frac{1}{\mu N \epsilon}. \tag{6}$$

This is independent of m and hence the total time to evolve the m-th mutant increases only linearly with m and not exponentially as in the uniform case (3). Drug gradients thus provide optimal conditions for the evolution of resistance: strong selection for more resistant mutants, and free resources (space and nutrients) to explore by successful genotypes.

Whether the absolute time for any given m will be larger or smaller in the gradient than in the uniform case depends on the population size N at the front from which new mutants emerge. In the case of steep gradients, N can be very small and gradients may actually slow down evolution compared to the uniform distribution of the drug. In addition, our reasoning breaks down if a mutation $m \to m+1$ lowers the resistance instead of increasing it. In this case the genotype $m+1$ almost never fix – the probability of such event is exponentially small in N [50]. The only way for the population to move forward is to produce a double (or higher) mutant $m \to m+2$ which has a positive fitness advantage over genotype m. The rate at which this happens is proportional to $\mu^2 N$ and, as μ^2 is very small, the adaptation time is determined mainly by this process and reads $1/(\mu^2 N)$. Since the effective population size N is smaller in the non-uniform case, drug gradients are likely to slow down evolution if a fitness valley is present in the mutational pathway.

5 Real infections

We shall now discuss how theoretical considerations presented above apply to *de novo* evolution of resistance in real infections. We shall consider two generic

scenarios: (i) a single mutation substantially increases resistance, (ii) multiple mutations are necessary to increase the MIC above the drug concentration achieved during a typical therapy. In (i), resistance will evolve as long as the population is large enough at the onset of treatment. In a large population, resistant mutants are always created even in the absence of the drug but they do not have any selective advantage over the sensitive strain and can grow to high abundance only after treatment has begun. We shall see that this may be the case for chronic infections in cystic fibrosis (Sec. 5.1), but this scenario may actually be quite common since bacterial densities often reach 10^8/ml in soft-tissue infections [35]. Such high densities diminish drug activity, sometimes by 100x, which lowers the required level of resistance and makes AMR easier to evolve [43].

Case (ii) requires a sufficiently slow increase of drug concentration at the site of infection so that multiple mutations have enough time to fix (Secs. 3.2 and 4). The critical time $T_{\rm crit}$ to evolve a more resistant mutant must be much smaller than the time $T_{\rm MIC}$ it takes the antibiotic to reach the MIC of the parent strain. This amounts to the following condition:

$$T_{\rm crit} \ll T_{\rm MIC}, \tag{7}$$

where $T_{\rm crit}$ is given by Eqs. (4,5). If the short-time evolution of the drug concentration is affected mostly by diffusion from a source such as a small blood vessel (Fig. 5), this can be rewritten as

$$T_{\rm crit} \ll \frac{x^2}{D\ln(c_0/{\rm MIC})}, \tag{8}$$

where x is the distance to the infection site, D is the diffusion constant and c_0 is the source (blood) concentration of the antibiotic. According to this estimate, *in vivo* evolution of drug resistance mediated by more than a single mutation is likely if: (i) the distance x is large, (ii) the drug diffuses slowly into the tissue (small D), (iii) bacteria replicate fast (large g), (iv) mutation rate μ is large. The last two conditions enter Eq. (8) via Eqs. (4,5).

Are these requirements fulfilled in a normal tissue? In a healthy and well vascularized tissue, the inter-capillary distance is about $100-200\mu$m [14], and so the infection site cannot be further than $x\approx 100\mu$m from the nearest capillary (drug source). The estimates on the diffusion constants of antibiotics in tissues vary [44, 45], but the lower limit is $D=10^{-7}{\rm cm}^2/{\rm s}=10\mu{\rm m}^2/{\rm s}$ [4]. The mutation probability μ ranges from 10^{-9} to 10^{-7}, depending on the antibiotic and bacterial strain [37, 51]. Fastest-reproducing bacteria have $g\sim 10^{-3}{\rm s}^{-1}$ [57]. Using Eq. (5) (relevant for sequential fixation) and inserting all these estimates into Eq. (8) we obtain that the condition is not fulfilled by a large margin. It is thus very unlikely to evolve drug resistance through

[4] Note that this value is much less than D measured in water or agar-based gels often used to study the diffusion of biomolecules [45].

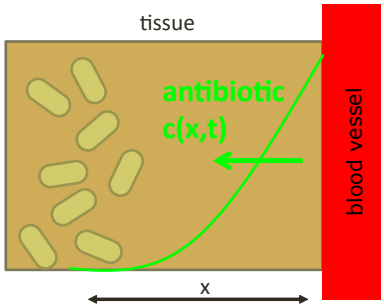

Fig. 5 A cartoon view of an infected tissue. The antibiotic diffuses with diffusion constant D from the blood where its concentration is c_0.

the above mechanism in normal tissues. However, not all sites of microbial infections are well vascularised and we shall see an example of that in Sec. 5.2.

5.1 Quasi-uniform drug distribution - P. aeruginosa infection in cystic fibrosis patients

Cystic fibrosis (CF) is a genetic disease characterized by thicker-than-normal secretions such as digestive fluids or mucus. CF affects mostly (but not exclusively) the lungs. The clinical picture is characterised by the presence of thick mucus plugging small and medium-size (0.3-5mm) bronchioles in the lungs - the tubes that carry air into pulmonary alveoli where gas exchange takes place. CF patients ofter suffer from lung infections caused by many pathogens, of which *P. aeruginosa* is the most significant one, accounting for 80% of chronic infections in adult CF patients [24]. Bacteria live in the mucus, where they form microcolonies and biofilms [58], with doubling times 100-200min [65] and densities up to $10^7 - 10^8$ bacteria per ml of mucus [6].

There is some evidence that a prolonged course of antibiotics can lead to *de novo* AMR evolution in chronic infections in CF patients [24]. In fact, the most common mechanism of resistance to aminoglycosides such as tobramycin (a popular drug used to treat *P. aeruginosa* infections) is decreased membrane permeability or increased activity of efflux pumps [54], both of which can be caused by point mutations [63, 64].

To estimate the likelihood of *de novo* evolution in a CF patient, we carried out computer simulations of a typical treatment against *P. aeruginosa* using the same model as in Sec. 3.1. We modelled a chronic infection with bacteria replicating with max. rate 0.34h^{-1} before treatment and being removed with rate $d = 0.27$. Treatment begun at $t = 0$ and consisted of 2 inhalations of 300mg tobramycin per inhalation per day [24]. We assumed that immediately

after application, the concentration of tobramycin was 20ug/ml [5] and its decay followed a 1st-order kinetics with half-life time 2h [6]. We also took the mutation probability to be $\mu = 10^{-9}$, the rate of point mutations in the PAO1 strain of *P. aeruginosa*. Following the experimental evidence from Ref. [63] we assumed that only one such mutation was required to increase the MIC from $\sim 1\mu$g/ml to $\sim 10\mu$g/ml.

The population was first allowed to grow to size N starting from a single cell and at the onset of treatment it consisted almost exclusively of sensitive cells (Fig. 6). A small sub-population of resistant cells was typically present at $t = 0$ but as these cells did not have any selective advantage, their number was low. However, during therapy ($t > 0$) in which sensitive cells had been killed, resistant cells were able proliferate and re-establish the infection after some time (Fig. 6, top). Fig. 6, bottom, shows that the regrowth probability was very small if the initial size $N < 10^8$, but it approached 100% for $N > 10^{10}$ cells – a number expected in about 100ml of mucus.

In reality, the situation may be worse (for the patient) than in our simulations. First, higher mutation rates (100x the basal rate) have been reported in *P. aeruginosa* isolates from CF patients [51]. Second, part of the population may be screened from the drug by forming a biofilm [61, 24, 54]. Third, bacteria can develop adaptive, non-genetic resistance to antibiotics [6]. This type of resistance caused by a temporal reduction in drug uptake [54] is unstable and bacteria revert to the sensitive phenotype when the antibiotic is withdrawn, but it can enable them to evolve genetic resistance by providing a temporary "safe niche" [38].

Finally, there may be spatial inhomogeneities in the distribution of the drug in the lung. Although difficult to model computationally in a realistic way, such heterogeneities will certainly play a role if a biofilm is formed, or when drugs with low diffusion rates are used. This may be particularly relevant to new drug classes such as antimicrobial peptides [66] which are bigger than many antibiotics and hence diffuse slower.

5.2 Drug gradients - infections of avascular tissues

We shall now consider a situation in which the distance x from the infected site to the source of antibiotic is much larger than 100μm. As explained above, the distance between capillaries which deliver blood (and drug) to the tissue is $\approx 100\mu$m and hence long-lasting antibiotic gradients do not occur in well-perfused, normal tissues. However, if blood circulation is impaired, large,

[5] We assumed that the concentration was 5x higher than the experimentally determined $3 - 5\mu$g/ml, following a single dose of 80mg [6]. The actual concentration may be lower – drug binding to serum proteins lowers the effective concentration of the drug, see e.g. [56, 8]. Ref. [48] claims 8μg/ml in the plasma, and sputum concentrations can be even lower [31]. All this can promote the evolution of resistance.

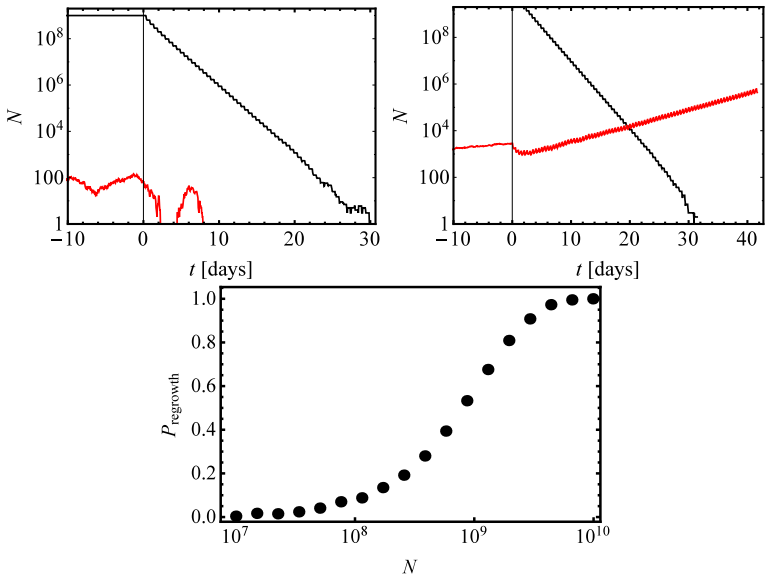

Fig. 6 Size and composition of a simulated microbial population following a course of tobramycin. Left: population size $N = 10^9$ at the onset of treatment. Right $N = 10^{10}$. Bottom: regrowth probability, equal to the fixation probability of the resistant mutant, for different N.

cm-size avascular necrotic areas can develop in which dead cells decay and become a rich source of nutrients fuelling microbial growth. This is common in chronic wounds, especially in the elderly who often develop pressure sores or ulcers due to poor mobility or underlying conditions such as diabetes [47, 49]. Bacterial infections of such wounds are common and are treated with both topical (antibiotic applied directly to the wound) and systemic therapy (antibiotic taken orally/intravenously) [40].

Although *de novo* AMR evolution is thought to be rare [49], the following numerical simulation demonstrates that it could easily occur in areas with deep necrosis or scarring. We model a 1cm^3 of ill-perfused tissue inhabited by 10^5 bacteria per mm^3 [47] at the onset of treatment. We use the same model as in the previous section[6] but we subdivide the tissue into $\sim 200\mu$m-size regions, each treated as a well-mixed system but with restricted migration between the regions (migration rate per bacterium $= 1\text{h}^{-1}$). This leads to a saturated colonization in about 5 days. We take *E. coli* as the pathogen and ciprofloxacin as the drug - though neither of them is the most common pathogen/therapeutic drug in wound treatment, ciprofloxacin has been used to treat soft tissue and wound infections [59, 20] and *E. coli* can be

[6] We take the maximal growth rate $g = 0.34\text{h}^{-1}$ and a lower removal rate $d = 0.1$ than before because the tissue is not perfused and bacteria are less likely to be removed by the immune system.

isolated from wounds [12]. Our choice is motivated by data availability: the nature of mutations and their effect on resistance have been well characterised for this particular combination [42, 29]. We assume the sensitive strain has MIC=30ng/ml, and two subsequent mutations increase the MIC to 300 and 3000 ng/ml, respectively.

Treatment begins when the total bacterial count is about 10^8. Ciprofloxacin diffuses into the system from the top side where its concentration is 1μg/ml - a typical concentration found in tissues and necrotic areas during treatment [17, 3]. Figure 7 shows that resistance evolves if the antibiotic cannot penetrate the tissue very well (diffusion constant $D = 10^{-7}$ cm^2/s) but not if the tissue is well vascularized and the antibiotic quickly reaches a uniform and high concentration.

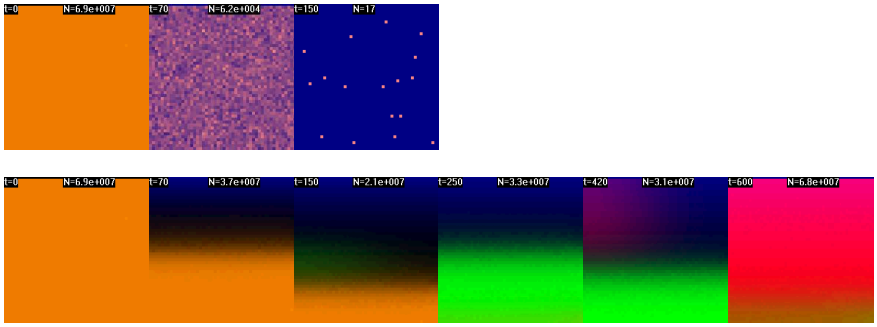

Fig. 7 Snapshots of a simulated infected tissue. Time is in hours, N is the total bacterial count. The concentration of the antibiotic/bacterial density is shown as different shades of blue/orange, respectively. Top: the tissue is well perfused and the antibiotic diffuses quickly. Bacteria are eradicated after approx. 70h. Bottom: avascular tissue, diffusion constant $D = 10^{-7}$ cm^2/s. After an initial decline, the bacterial population grows back due to the evolution of resistant mutants (green and red).

6 Summary

We have reviewed here the theory and recent experiments on the evolution of AMR. We have focused on the population dynamics and the role of spatial heterogeneities in drug distribution. Many models of bacterial infections and treatment were proposed in the past (see e.g. [5, 39, 10]) but they usually treated all resistant genotypes as a single species. Here we have studied the role of the spectrum of mutants on AMR evolution. We have shown that if a sequence of mutations progressively increases the resistance, a highly-resistant strain will often evolve faster if the drug is non-uniformly

distributed. We hypothesize that this may be the case in some soft tissue infections.

Although we have not explored this avenue here, it is also likely that *de novo* evolution takes place in biofilms [19], where drug gradients can be stronger than in tissues due to decreased permeability and active degradation of the drug. Although resistance in biofilms often relies on non-genetic alterations [61] such as decreased permeability or switching to a different phenotype, these alterations may actually promote genetic evolution through the "save niche" mechanism mentioned in Sec. 5.1.

Further theoretical and experimental research may address AMR evolution in the presence of interactions between different drugs [15, 9], and the role of social interactions between the microbes such as reported in a recent work [34] on *de novo* evolution of vancomycin-intermediate resistant *S. aureus*.

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