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Non-Darwinian Selection of Plasmid-Mediated Antibiotic Resistance

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ABSTRACT

Resistance of bacteria to antibiotics is an inevitable consequence of long-term exposure to sub lethal drug concentrations, and the problem has been greatly exacerbated by inappropriate use. Resistance can be conferred by chromosomal mutations that are passed on in normal cell division (vertical gene transfer) or by horizontal gene transfer processes such as conjugation. Darwinian natural selection implies that individual organisms possessing a survival advantage over their peers will represent an increasing proportion of the total population in subsequent generations. Using the techniques of evolutionary dynamics, we have modelled the selection of antibiotic-resistant strains of bacteria resulting from either vertical or horizontal gene transfer, and the consequences of these events for drug sensitivity. Our model suggests that the dynamics of horizontal gene transfer are such that plasmid-bearing bacteria may dominate a population despite having a growth disadvantage compared to the same bacteria that do not carry the plasmid. Horizontal gene transfer than by vertical transfer propagates antibiotic resistance more rapidly. These findings have implications for the design of combination drug regimens for treatment of bacterial infections.

Keywords

Antibiotic resistance, Evolutionary dynamics, Horizontal gene transfer, Non-Darwinian selection, Plasmid dynamics.

Abbreviations

DT: Doubling Time, GF: Growth Fraction, GLK: Gross Log Kill, HGT: Horizontal Gene Transfer, ILS: Increase in Lifespan, MTD: Maximum Tolerated Dose, TI: Therapeutic Index.

Introduction

The development of acquired resistance to antibiotics is inevitable, because random mutations will occasionally throw up variant organisms that possess a selective growth advantage in the presence of a drug. However, the incidence of resistance has been greatly increased by such practices as over-prescribing, prescribing for inappropriate diagnoses, self-medication and use in animal husbandry. Many commentators predict that if this problem cannot be solved, society will face a return of large-scale epidemics of bacterial diseases, for which we shall have no effective treatments. Moreover, since most surgical procedures are critically dependent on infection control, widespread antibiotic resistance will return the safety of surgery (and organ transplantation and cancer therapy) to the pre-antibiotic era. The UK Prime Minister has commissioned a report on this problem [1] and its conclusions have been widely reported in the lay press [2,3].

Horizontal gene transfer (HGT) is believed to have played an important role in bacterial evolution [4,5]. Among the consequences of this mode of genetic information, transfer is rapid spread of infectious disease [6]. In addition, plasmid genes tend to evolve more rapidly than chromosomal genes [7]. Because it can occur between bacteria of different species, HGT facilitates the colonization of different host species [8]. Mechanisms of HGT include conjugation (in which genetic information carried on a plasmid is transferred from one bacterial cell to another), transformation (in which a bacterial cell takes up DNA molecules from the extracellular medium) and transduction (which is phage-mediated). Horizontal transfer of drug resistance genes is believed to occur primarily by conjugation [9]. In many cases, including gram-negative organisms such as E. coli, Klebsiella, Acintebacter and Pseudomonas spp. resistance may be both chromosomal and plasmid-mediated.

Raz and Tannenbaum [10] modelled the conjugation dynamics of bacteria and concluded that the advantage of HGT was its ability to promote faster adaptation in dynamic environments. They noted that this interpretation was consistent with the observation that HGT can be promoted by environmental stresses on a population. The form of environmental stress that we are concerned with in the present discussion is the presence of antibiotics. A major difference between the evolutionary dynamics of point mutations and HGT is that the former occur spontaneously, whereas HGT requires a pre-existing donor cell. This need not be a cell of the same species: plasmids may be passed between bacteria of different species. It has been shown that non-pathogenic bacteria may provide a reservoir of antibiotic resistance plasmids without any ill effect on the host until the plasmid is passed to a previously drug-sensitive pathogen [11].

The quantitative study of evolutionary dynamics began with the description by Luria and Delbrück of phage resistance in bacterial cultures [12]. They related the number of bacteria that were resistant to phage destruction, μ, to the total number of bacterial cells, N, and two constants: α, the mutation rate for conversion from phage sensitivity to resistance, and β, the reversion rate of resistant bacteria back to phage sensitivity:

$$\mu = \alpha N(1 - N^{-\alpha-\beta}) / (\alpha + \beta) \qquad \text{(Equation 1)}$$

Goldie and Coldman [13] working with transplanted tumours in inbred mice showed that these tumours could be cured with chemotherapeutic agents so long as no pre-existing drug-resistant cells were present at the start of treatment. A single drug-resistant cell could result in failure of treatment and the larger the tumour burden at the start of treatment, the more likely it was that drug-resistant mutant cells would be present and result in treatment failure. Goldie and Coldman worked with syngeneic tumour cells in inbred mice, so that there was no anti-tumour immune response. Subsequent investigators extended this analysis to human tumours, and showed that curative cancer chemotherapy usually required drug combinations, since the probability of double mutants was low enough that they would not be present at the start of treatment. For large tumours, three-drug combinations were required [14]. An important conclusion of these studies was that simultaneous treatment with multiple drugs was superior to sequential treatment. If a single drug is used until, the tumour no longer responds, the probability that cells resistant to a second drug will be already present when treatment with the second drug is started will be greatly increased [15]. Similar considerations will apply to the curability of bacterial infections by antibiotic combinations when antibiotic resistance is chromosomal and vertically transmitted. The goal of the present study was to determine whether HGT changes the dynamics of antibiotic resistance in ways that have implications for the design of curative treatment.

There are similarities and differences between the evolutionary dynamics of bacteria and that of cancer cells. Mutation rates in bacteria are relatively low compared to cancer cells, which are genetically unstable. On the other hand, because of their rapid proliferation rate, a bacterial infection at the time of diagnosis may have a much higher cell count than a tumour, raising the probability of pre-existing resistant cells. The carrying capacity of the human body for bacteria in the colon may be as high as 10^{14} cells, whereas for cancer 10^{12} cells would usually represent a lethal tumour burden. By contrast, the bacterial count in a bladder infection may be about 3×10^8. The other major difference in the dynamics of bacterial disease is that bacteria are highly immunogenic. This means that to cure the disease, the drugs do not have to reduce the pathogen count to zero. Within hours of an infection, the innate immune system will result in the infected tissue being invaded by macrophages and neutrophils. Within a few days, an immunocompetent individual will mount a strong antibody response, and after the infection has been cleared, a small fraction of the activated B-lymphocytes and T-lymphocytes will persist as memory cells, to provide a rapid response in the event of a subsequent infection. An evolutionary dynamics model of bacterial disease must describe all these phenomena. The evolutionary dynamics of intracellular pathogens differs from that of bacteria growing in extracellular fluid. The present study considers only extracellular organisms.

Methods
Model Description
Cell proliferation is treated as Gompertzian [16]. The Gompertz equation describes the situation where growth starts out exponential, but where the growth rate itself declines exponentially as resources are depleted:

$$dN/dt = kN/c \;.\; (A - \log_e N) \qquad \text{(Equation 2)}$$

N is cell number, and k is the growth rate constant = $\log_e 2$/initial doubling time. A is a function of the asymptote, the final cell count at which growth levels off (also known as the carrying capacity). If the final cell count is represented as N_{inf} (N at time infinity) then

$$A = \log_e N_{inf} \qquad \text{(Equation 3)}$$

Thus, $(A - \log_e N)$ is a measure of how far the system is, at time t, from its final value. Similarly, the constant c is a measure of how far the final state is from the initial cell number, N_0:

$$c = A - \log_e N_0 \qquad \text{(Equation 4)}$$

Gompertzian growth implies that a fraction of each population will be non-proliferating, and non-proliferating bacteria tend to be harder to treat [17,18]. The growth fraction (GF) is the proportion of the total bacterial population that is proliferating at a particular time:

$$GF = A - \log_e(N_t) / c \qquad \text{(Equation 5)}$$

The number of quiescent (dormant) cells at time t is then $N_t . (1 - GF)$

The model assumes that, depending on the organism and on the site of the infection, there will be a maximum number of bacteria that the host can tolerate, and bacterial growth beyond this number will be lethal. In general, the lethal bacterial count will be lower than the theoretical carrying capacity, though this is not a condition of the model. If the carrying capacity for an organism is lower than the lethal count, the infection will persist as a chronic condition.

Mutations occur by inaccurate base pairing during the replication process. During any time, dt, the number of instances of a particular mutation will be:

$$(N_{t+dt} - N_t) \cdot m \qquad \text{(Equation 6)}$$

Where m is mutation rate.
Reversion, i.e. back mutation from drug resistance to drug sensitivity, is also described by equation 6. The reversion rate is not necessarily the same as the forward mutation rate.

Horizontal gene transfer is a second-order process:

$$v_{hgt} = N_s \cdot N_r \cdot k_{hgt} \qquad \text{(Equation 7)}$$

Where N_s and N_r are numbers of drug-sensitive and -resistant cells, respectively, k_{hgt} is a second-order rate-constant and v_{hg} is the rate of horizontal gene transfer, cells transfected per unit time.

Plasmid loss may occur when a bacterial cell divides, and plasmid replication has not kept pace with chromosomal replication, or when chromosomal DNA and plasmid DNA do not segregate together during cell division. The model treats plasmid loss as a first-order process.

Plasmids present a cost to their host cell, because additional DNA has to be replicated. There is a relationship between plasmid size and cell doubling time [19]. In the model, the cell doubling time of plasmid-bearing cells may be longer than for wild-type cells.

Pharmacodynamics
Drug effects may be cytotoxic or cytostatic. Antibiotic cytotoxicity dose-response curves are described by a semi-logarithmic relationship [20]:

$$\text{Cell kill, ck (log)} = \text{dose/MTD} * \text{GLK} / \text{Rfactor} \qquad \text{(Equation 8)}$$

Where MTD = maximum tolerated single dose (mg/kg)
GLK = gross log kill of bacteria at MTD (i.e. not corrected for re-growth of surviving cells during the treatment period). Rfactor = resistance factor, defined as GLK for resistant organism/GLK for wild type. Then, for a cytotoxic drug:

$$N \text{ (treated)} = N \text{ (control)} / 10^{ck} \qquad \text{(Equation 9)}$$

Cytotoxic effects may be selective for dividing cells, or active against both replicating and non-replicating cells.

For a cytostatic drug, the doubling time (DT) for cell replication is increased. The dose-response curve is described by a Hill equation [16]:

$$\text{DT (treated)} = \text{DT (control)} * (1 + \text{Imax} * (\text{dose/MTD})^{nH}/(1+ (\text{dose/MTD})^{nH})) \qquad \text{(Equation 10)}$$

Where IMAX is the maximal growth-inhibitory effect and nH is the Hill coefficient.
Cytotoxicity to normal mammalian tissues is calculated by the same equation, except that the therapeutic index (TI) is substituted for the resistance factor

$$N \text{ (treated)} = N \text{ (control)} / 10^{k'} \qquad \text{(Eequation 11)}$$

$$\text{Where } k' \text{ (log)} = \text{dose/MTD} * \text{GLK} / \text{TI} \qquad \text{(Equation 12)}$$

Survival advantage is then a balance of selection pressure, proliferation rate and death rate. Plasmids can only be maintained in a population when the rate of horizontal gene transfer is larger than the combined effect of segregational loss and the decrease of fitness associated with plasmid carriage [21].

Programming
The computer implementation of our model, neoMYCIN, is coded in C. Source code is included in the supplementary material. It was compiled using GNU Compiler Collection (gcc) release 4.6.2. The equations of the model are listed in the supplementary material. Plots were generated using gnuplot [22].

Results
Our simulations suggest that acquired drug resistance is a major cause of treatment failure in humans. Figure 1 shows the predicted response of a *Pseudomonas aeruginosa* infection to eight daily treatments with a maximum tolerated dose of imipenem (3500 mg). The untreated infection reached a lethal bacterial count in under 3 days (Figure 1, control curve). Assuming that there were no drug resistant cells, eight days of treatment eliminated the infection.
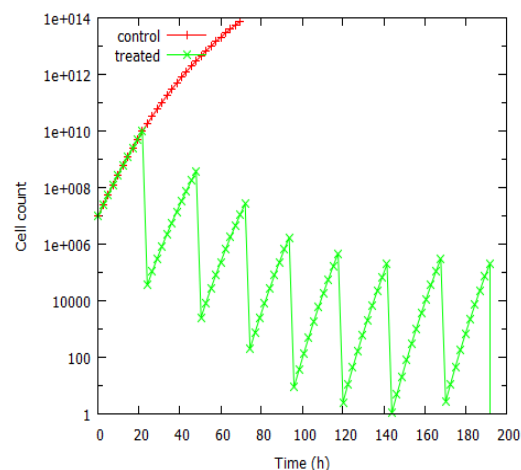

Figure 1: Curative Treatment with Eight Daily Doses of Imipenem in the Absence of Drug-Resistant Cells.

Figure 2 models the situation where resistance emerges to this treatment regimen with a mutation rate of 8.0 x 10^{-7} per cell division. The bacterial count initially responded, but by 72h, most of the bacteria were drug resistant and the bacterial count reached a lethal level in less than seven days. In this simulation, the initial number of resistant cells was calculated from the equation of Luria and Delbrück [12]. If it was assumed that there were no resistant cells present initially, it took several days for resistance to emerge, but the qualitative outcome was the same.
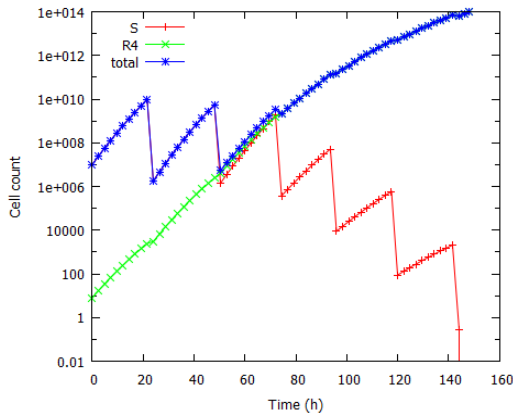

Figure 2: Effect of Vertically Transmitted Antibiotic Resistance.

If it was now assumed that the gene causing antibiotic resistance was plasmid-borne, and thus vertically transmitted, the outcome was as shown in figure 3. The number of resistant cells present initially was the same as for figure 1. By 48h, resistant cells dominated the population, and the infection reached a lethal bacterial count within 5 days.
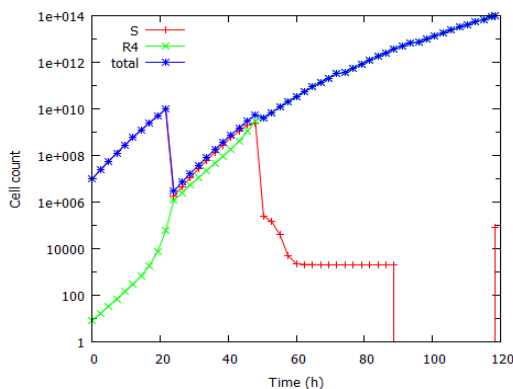

Figure 3: Effect of Horizontally Transmitted Antibiotic Resistance.

In the absence of selection pressure, chromosomal drug resistance genes tend to be spontaneously eliminated. Subsequent simulations were directed to determining whether the same considerations apply to horizontal transfer of resistance. We thus attempted to predict plasmid spread in presence and absence of drug and the extent and effect of spontaneous plasmid loss. Does the extra DNA of a plasmid-bearing bacterial cell represent a competitive disadvantage? If we calculate the cell dynamics assuming vertical

gene transmission and Darwinian selection, the resistant cells must have a competitive disadvantage in the absence of selection pressure: if it were otherwise, the resistant cells would *be* the wild type. Our model indicates that with horizontal gene transfer, even with a large growth disadvantage, even in the absence of drug, plasmid-bearing bacteria will still eventually dominate the population. Figure 4 shows the growth of drug-sensitive bacteria (S) and a drug-resistant variant (R4) who is doubling time is twice that of the wild-type cells. The drug-resistance is assumed to result from a chromosomal mutation. The two populations start out the same, but because of their longer doubling time, the resistant cells are outgrown, and within a few days, the count of resistant cells is four logs lower than that of the wild type. Figure 5 shows a similar calculation in which all parameter values are the same, except that drug-resistance is now plasmid-mediated. In this case, within 66 hours >99.9% of the bacteria are plasmid-transfected and therefore drug-resistant.
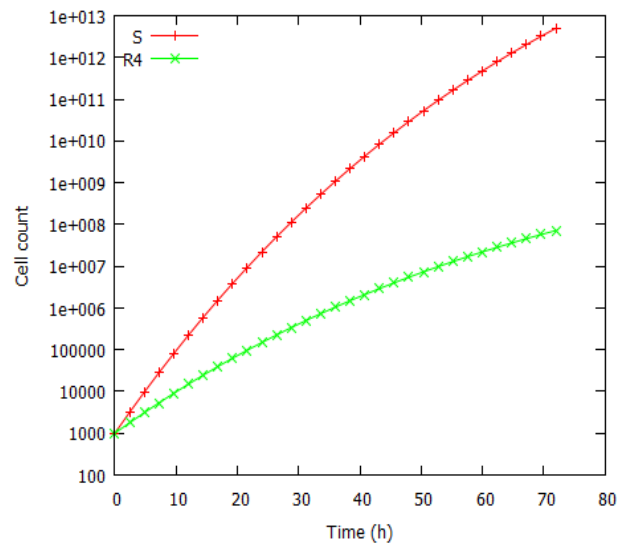

Figure 4: Growth of Drug-Sensitive (S) and -Resistant (R4) Cells in the Absence of Selection Pressure
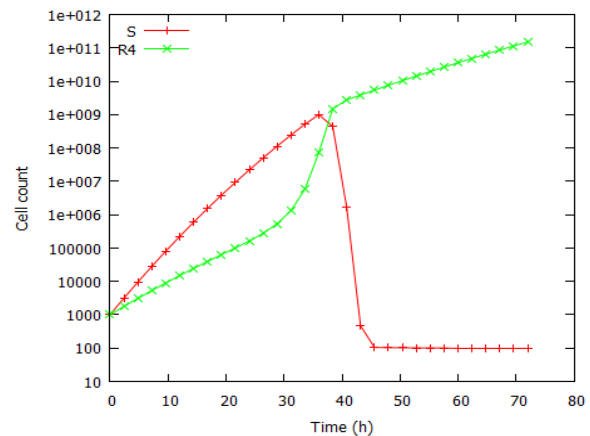

Figure 5: Growth of Drug-Sensitive Bacteria (S) and Bacteria Carrying a Drug-Resistance Plasmid (R4) In the Absence of Selection Pressure
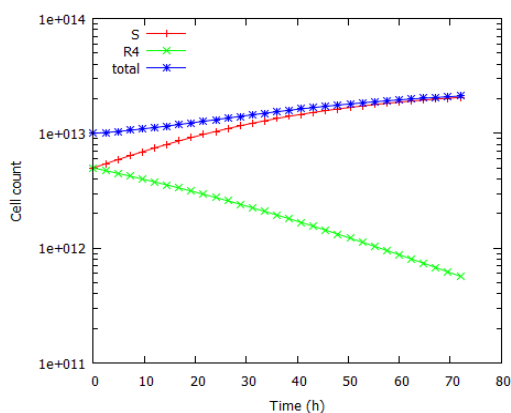
Figure 6: Selection against Drug-Resistant Bacteria, In Absence of Drug, and When Resistance is Vertically Transmitted.
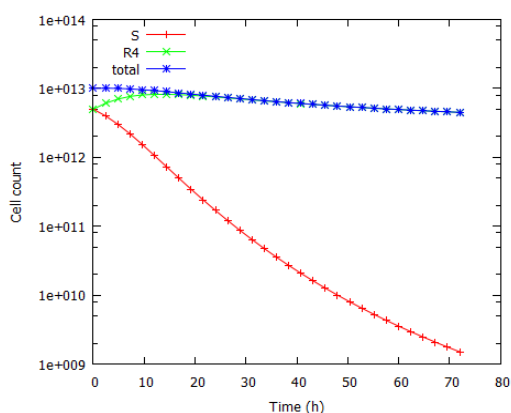

Figure 7: Selection in Favour of Drug-Resistant Bacteria, in Absence of Drug, When Resistance is Horizontally Transmitted.

The usual approach to preventing antibiotic resistance-related treatment failure is to use combinations of non-cross-resistant drugs. The rationale is that cells resistant to drug A will be susceptible to drug B, and vice versa. If the incidence of resistance to drug A is a, and to drug B is b, the incidence of double mutants resistant to both drugs will be $a.b$. Our model has been used to model many combinations. Initially, we assumed that antibiotic resistance genes were carried on the bacterial chromosome. Table 1 summarises a number of such simulations. The activity of single agents was always transient and limited by the emergence of resistance. All three two-drug combinations gave improved response durations, but all eventually resulted in overgrowth of doubly resistant cells. However, a three-drug combination was curative in this system.

To demonstrate the consequences of plasmid-mediated drug resistance for treatment of advanced infection, we repeated the calculations of Table 1, using the same parameter values, except that resistance to one of the drugs, imipenem, was now assumed to be caused by a plasmid. Results are shown in Table 2, and are generally similar to those of Table 1. A few differences were apparent: when imipenem resistance was caused by a chromosomal

mutation, as in Table 1, imipenem-resistant cells only appeared in presence of the drug. However, Table 2 shows that cells transfected by the plasmid carrying imipenem resistance (R4 in Table 2) usually became the dominant population, even when no imipenem was present. In several cases, multidrug-resistant cells dominated the population, but these also carried the plasmid. The other notable difference was that the three-drug combinations, including the combination that was curative in Table 1, were now unable to eliminate the infection, though a four-drug combination was able to do so.

Table 1: Predicted Effects of Treatment of an Advanced *Pseudomonas Aeruginosa* Infection with Single Drugs and Combinations, Assuming Resistance is Caused by Mutations in Chromosomal Genes.

Tobramycin	Ceftazidime	Imipenem	Survival (d)	ILS (%)	Failure from:
0	0	0	1.5	0	S
350	0	0	2.8	87.9	R1
0	6000	0	3.8	157.8	R2
0	0	2000	3.7	141.5	R4
350	6000	0	8.1	444.2	R12
350	0	2000	7.6	411.5	R14
0	6000	2000	8.5	471.6	R24
350	6000	2000	cure at 4.0 d.		

Doses in mg/day for up to 14 d.
ILS = increase in lifespan. Inoculum was 5 x 10⁹ cells
S: wild-type (drug-sensitive bacteria).
R1: bacteria resistant to tobramycin
R2: bacteria resistant to ceftazidime
R3: bacteria resistant to ciprofloxacin
R4: bacteria resistant to imipenem
R12: double-mutants resistant to tobramycin and ceftazidime
R14: double-mutants resistant to tobramycin and imipenem
R24: double-mutants resistant to ceftazidime and imipenem

Table 2: Predicted Effects of Treatment of an Advanced *Pseudomonas Aeruginosa* Infection with Single Drugs and Combinations, Assuming Resistance to Drugs 1, 2 and 3 is caused by Mutations in Chromosomal Genes and Resistance to Drug 4 Is Plasmid-Mediated.

Tobramycin	Ceftazidime	Ciprofloxacin	Imipenem	ILS (%)	Failure from:
0	0	0	0	0	R4
350	0	0	0	87.9	R4
0	6000	0	0	157.8	R4
0	0	1500	0	73.2	R4
0	0	0	2000	39.7	R4
350	6000	0	0	613.3	R124
350	0	1500	0	370.2	R4
350	0	0	2000	158.3	R4
0	6000	1500	0	492.7	R24
0	6000	0	2000	299.3	R24
0	0	1500	2000	138.6	R4
350	6000	1500	0	1184.5	R124
350	6000	0	2000	1134.8	R4
350	0	1500	2000	863.2	R14
0	6000	1500	2000	1050.8	R4
350	6000	1500	2000	cure at 2.0 d	

Abbreviations as for table 1.

Discussion

In a study of the dynamics of genes involved in bacterial social behaviour, McGinty et al. [23] showed that horizontal gene transmission results in increased frequency of plasmid-borne social goods. By "social goods", they meant properties such as virulence, immune evasion and biofilm formation that confer advantages on the entire population. Similar considerations apply to the spread of antibiotic resistance.

How do the dynamics of HGT resistance differ from chromosomal resistance? Chromosomal resistance is spontaneous, and given a sufficiently large bacterial load, it is therefore inevitable. In contrast, plasmid-mediated resistance requires a "seed" (a pre-existing plasmid-carrying cell), so it is in principle preventable by using strict isolation procedures. However, the risk of plasmid-mediated resistance is greatly increased by the fact that it can cross species barriers. Non-pathogenic bacteria, for example on the skin or in the gut, may harbour plasmids carrying genes for antibiotic resistance, and may pass them to previously drug-sensitive pathogens.

Plasmid-mediated resistance is autocatalytic. The requirement for cell-cell contact makes it a second-order process. The newly-transfected cell is now able to replicate the plasmid, and not only pass it on to its own descendants but also to transfect any remaining drug-sensitive cells through HGT. The dynamics of the conjugation process are such that it simultaneously increases the number of antibiotic-resistant cells and decreases the number of sensitive cells

Chromosomal resistance confers a selective disadvantage in the absence of selection pressure; the resistant cells tend to be less "fit" in the Darwinian sense. If that were not the case, they would have become the wild type. As we showed in figure 7, plasmid-mediated resistance, in contrast, can propagate in competition with sensitive strains even in absence of selection pressure, i.e. it is non-Darwinian. In the absence of plasmid loss, HGT will result in the population consisting entirely of resistant cells, even when they replicate more slowly than the wild type. This picture is complicated by the fact that plasmid loss does occur, and is likely to be greater for very large, multi-drug-resistance plasmids.

Plasmid-mediated resistance is more difficult to eliminate with combination therapy. Assuming a rather high average mutation rate of 1×10^{-6}, the probability of there being pre-existing triple mutants in a population of 10^{14} cells is negligible. It should be possible to cure any bacterial infection with a combination of three drugs, assuming that they are mutually non-cross-resistant, and that their host toxicities do not overlap, so that they can be administered at full doses (Table 1). This argument, assumes, of course, that all three drugs have significant, even if non-curable, single agent activity against the relevant pathogen. However, if resistance to at least one of the antibiotics is transmitted by HGT, this analysis no longer applies. In the example studied in Table 2, where imipenem resistance was plasmid-mediated, it was possible to eliminate the infection with a four-drug combination.

If a plasmid carries resistance genes for more than one antibiotic, as is commonly the case, the dynamics will be more complex. Moreover, for combinations of four or more drugs, the requirement for non-cross resistance and non-overlapping toxicities will be harder to meet. It will sometimes be necessary to settle for agents with partial cross-resistance, or drugs that have to be used at reduced doses. Our model can be used to evaluate the potential efficacy of large numbers of combinations. If population PK/PD data can be incorporated into the model, it will be possible to conduct *in silico* clinical trials. The ability to compare large numbers of clinical trial designs computationally is a tool that can be used to prioritise trial designs for experimental evaluation [16].

The dynamics of HGT suggest that agents that can inhibit the process would be a valuable addition to the pharmacopoeia. A number of new targets for inhibiting HGT are under evaluation [24-26].

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