

OPINION

Non-inherited antibiotic resistance

Bruce R. Levin and Daniel E. Rozen

Abstract | In addition to their impressive, well-publicized and well-researched propensity to evolve and acquire genetically determined mechanisms for resistance to antibiotics, bacteria that are inherently susceptible to these drugs can also be phenotypically refractory to their action. This phenomenon of 'non-inherited resistance' to antibiotics has been known since the beginning of the antibiotic era but, relative to inherited resistance, it has been given little attention. Here, we review the *in vitro* and *in vivo* evidence for the different forms of non-inherited resistance and the mechanisms responsible. With the aid of a simple mathematical model and computer simulations, we show how non-inherited resistance could extend the duration of antibiotic treatment, cause treatment failure and promote the generation and ascent of inherited resistance in treated patients.

Antibiotic resistance has been, and continues to be, a major focus for many applied and basic scientists. As of February 2006, PubMed listed nearly 66,000 publications in which the phrase 'antibiotic resistance' appears in 'any field', and more than 3,700 in the title. In almost all of these publications, the resistance of concern is that encoded by chromosomal genes and/or accessory genetic elements. There are good reasons for the attention given to inherited antibiotic resistance. It is a substantial health problem and shows every sign of getting worse^{1–5}. Inherited antibiotic resistance also has numerous characteristics that make it attractive to basic and applied researchers. In addition to being a good marker (tool) for genetic manipulations, it is amenable to epidemiological monitoring and surveillance studies and is the basis for interesting studies that focus on bacterial genetics, molecular biology, physiology, biochemistry and even mathematical modelling. For the pharmaceutical industry, inherited resistance to existing antibiotics has been a significant motivating force for the discovery and development of new drugs^{6,7} and compounds that overcome the mechanisms of resistance^{8–12}.

Although inherited resistance to antibiotics (a word we use here for all chemical antibacterial agents used for prophylaxis and treatment) has the appeal of clinical reductionism — a single factor to explain or excuse undesired outcomes of treatment — it is not the only reason antibiotics fail and, for many bacterial infections, it might not even be the main reason for treatment failure. Pneumococcal pneumonia is a good

example of this. The average rate of mortality of patients with bacteraemic pneumococcal pneumonia is currently 12–17% (REFS 13,14), and it has remained at that level for the nearly five decades since penicillin first became widely available for the treatment of this disease^{15,16}. Although the frequency of *in vitro* resistance to β -lactams and other antibiotics used to treat pneumococcal pneumonia has increased during this time¹⁷, inherited antibiotic resistance can account for little of this mortality^{13,14}.

It is clear that the course of an infection, and therefore the success of antibiotic treatment, depends on numerous host factors, including age, state of health and even genetics¹⁸. After all, antibiotics have only a supportive role in the control and clearance of bacterial infections. The morbidity and mortality of bacterial infections in mammalian hosts can be attributed not only to the direct effects of the endo- and exo-toxins and other virulence factors these organisms display and secrete, but also to the over-response of the host immune system to these bacteria and their products^{19–22}. As the duration (age) of an infection increases, and the direct and collateral damage owing to the proliferation of the bacteria accumulate, even the complete clearance of the bacteria responsible might have no effect on the clinical outcome of the disease²².

However, part of the reason that genetically susceptible bacteria treated with antibiotics are neither killed nor prevented from replicating can be attributed to their physiological state and the physical structure of their populations in infected hosts at

the onset of treatment. In this article, we review the mechanisms by which bacterial populations, or population fractions, that are genetically homogeneous and ordinarily susceptible to antibiotics become transiently refractory to their action, phenomena we refer to collectively as non-inherited resistance. In contrast to inherited resistance resulting from mutations in existing genes or the organism's acquisition of external resistance-encoding genes, non-inherited resistance is purely phenotypic. The population's contact with antibiotics does not change its inherent susceptibility to these drugs. Using a mathematical model and computer simulations we illustrate scenarios by which non-inherited resistance can affect the microbiological course of antibiotic treatment and ultimately lead to treatment failure. We conclude with an appeal for more research on the contribution of non-inherited resistance to antibiotic treatment failure and greater consideration of these forms of resistance in the design of antibiotics and treatment regimes.

Drug indifference

Not long after penicillin was first developed for clinical use, it became apparent that the rate at which bacteria were killed by this drug *in vitro* was directly proportional to their rate of growth²³. Bacteria that were not dividing were not killed by the drug. This phenomenon, which Walsh McDermott termed 'drug indifference' in an inspiring review of this subject, is not restricted to the β -lactam class of antibiotics^{24–26}. Bacteria that are not dividing and/or have insufficient nutrients for active metabolism are partially or completely refractory to killing by antibiotics from most, if not all, the major classes.

FIGURE 1 shows the susceptibility of growing populations of bacteria to antibiotics at bactericidal concentrations, and their absolute or relative indifference to these drugs when they are neither growing nor have resources for metabolism. In these experiments, *Escherichia coli* K12 strain MG1655 was exposed to bactericidal concentrations of antibiotic from four different classes. In one set of cultures, the bacteria were allowed to grow exponentially in nutrient broth. In the other set of cultures, stationary-phase bacteria were suspended in 1% saline (FIG. 1). The changes in the densities of viable bacteria in these cultures were estimated by diluting and plating on Luria Bertani agar.

All of the antibiotics are effective at killing exponentially growing bacteria. However, bacterial cultures in stationary phase survive equally well in the presence of

ampicillin and tetracycline when compared with their viability in antibiotic-free conditions. Although stationary-phase bacteria are killed to some extent by streptomycin and ciprofloxacin, their rate of mortality is substantially less when compared with the corresponding exponentially growing cultures. For further discussion of this phenomenon see REFS 27–29.

That drug indifference is not an artefact of *in vitro* culture was evident more than a half century ago from Harry Eagle's studies of penicillin treatment of experimental *Streptococcus pneumoniae* and *Treponema pallidum* infections in mice and rabbits^{30–32}. In these experiments, the concentration of penicillin required to 'cure' the infections increased with both the inoculum size and the duration of the infection. A significant reason, if not the sole reason, for this phenomenon is that as the infection progresses the rate of growth of the bacterial population seems to slow and then stop, possibly because of exhaustion of local nutrients. For this reason, the infection is refractory to the antibiotic treatment^{30,33–35}.

The declining efficacy of antibiotics over the duration of an infection can be readily observed using a 'resistance competition assay' (reconstruction experiments in which the inoculated population of bacteria includes a majority of cells that is susceptible to the administered antibiotic and a minority that is genetically resistant)^{36,37}. When the

infection is treated early, the resistant minority increases rapidly in frequency. Antibiotic-mediated selection confers high fitness to resistant cells owing to their higher rate of survival and replication in the presence of these compounds, relative to susceptible cells. When antibiotic treatment is delayed, the rate and extent of the increase in the frequency of the resistant minority is low or negligible, that is, the benefit to resistant cells is diminished. In an *E. coli* K1 mouse-thigh model of infection, when treatment with streptomycin is initiated more than 8 hours after the bacteria are inoculated, the streptomycin-resistant minority has virtually no advantage over the susceptible bacteria.

The effect of this phenomenon on the mortality of the mice is dramatic. In the absence of treatment at the bacterial inoculation densities used, *E. coli* K1 infections are almost invariably lethal^{36,38} with the mice dying in an average of 32 hours after infection. When a single 60 $\mu\text{g gm}^{-1}$ dose of streptomycin was administered immediately after the inoculation of mice with the bacteria, none of the 15 mice inoculated died of the infection. When this single-dose treatment with either 60 $\mu\text{g gm}^{-1}$ or 100 $\mu\text{g gm}^{-1}$ of streptomycin was delayed for 8 hours, 7 of the 12 and 9 of the 12 mice that were infected, respectively, succumbed to the infection³⁶.

Recently, Renata Zappala presented evidence that the relative inefficacy of streptomycin in these delayed-treatment

experiments can be attributed to the fact that after 8 hours, the bacteria in the host no longer divide³⁹. As a measure of cell division in these thigh-model experiments, Zappala used the decline in the fraction of the *E. coli* K1 population carrying a single-copy plasmid that did not replicate at temperatures above 32°C. Shortly after inoculation, the fraction of the population with this plasmid declined, indicating that the cells were dividing. But 8 hours after infection, this fraction remained effectively constant, indicating that bacterial cell division had ceased.

The contribution of non-replicating (dormant) antibiotic-refractory, but genetically susceptible, subpopulations of bacteria to the efficacy of antibiotic treatment is particularly well known for the treatment of infections with *Mycobacterium tuberculosis*^{40,41}. This latency is the primary reason for the long duration of what is called 'short-course' tuberculosis chemotherapy. Latent and residual populations of non-growing bacteria could also contribute to relapses following the cessation of antibiotic treatment for mycobacterial, staphylococcal and other bacterial infections^{41–44}.

Persistence

Since the early days of the antibiotic era it has been known that even when bacteria have sufficient resources for metabolism and growth, bactericidal antibiotics do not kill all of the bacteria in an actively dividing culture⁴⁵. As time proceeds, the rate of killing declines and substantial fractions of the bacterial population survive their encounter with these drugs (FIG. 1a). This phenomenon has been known as 'bacterial persistence'^{45,46}, 'adaptive resistance'⁴⁷ and 'phenotypic tolerance'^{48,49}. For historical reasons, and to avoid ambiguity with other forms of non-inherited resistance, we shall refer to this decline in the rate of antibiotic-mediated mortality in dividing bacterial populations and the subsequent survival of a fraction of the population as persistence. It differs from the drug indifference that is induced by starvation of essential nutrients in that, at any given time, only a small fraction of a dividing bacterial population (the persistent cells) is refractory to killing by the antibiotic. Although the capacity to generate persistent cells is a genetically determined property of a bacterial population, persistence itself, like population-wide drug indifference, is a non-inherited form of resistance — the persistent-cell subset of the antibiotic-exposed population is genetically identical to the cells that have been killed by the antibiotic. When these surviving, persistent cells are grown in

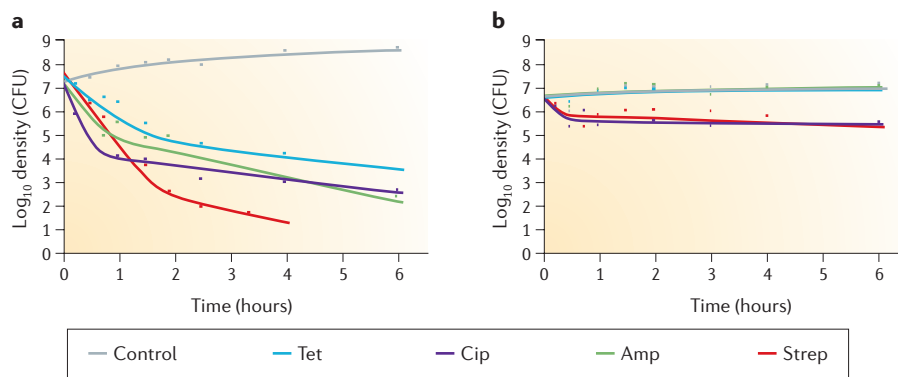
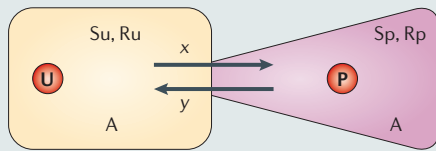


Figure 1 | Viability of exponential-phase and stationary-phase bacteria in the presence of antibiotics. The change in the viable cell densities of exponential-phase and stationary-phase bacteria (*Escherichia coli* K12 strain MG1655) exposed to 128 $\mu\text{g ml}^{-1}$ ampicillin (Amp), 64 $\mu\text{g ml}^{-1}$ tetracycline (Tet), 1 $\mu\text{g ml}^{-1}$ ciprofloxacin (Cip), 128 $\mu\text{g ml}^{-1}$ streptomycin (Strep) and in the absence of antibiotics (Control). The minimum inhibitory concentrations of these drugs for the bacteria, estimated by serial dilution, were 2.0, 2.0, 0.06 and 8.0 $\mu\text{g ml}^{-1}$ for Amp, Tet, Cip and Strep in that order. The lines are fitted. **a** | Growth and mortality of *E. coli* K12 MG1655 in Luria Bertani (LB) broth. Overnight LB cultures of bacteria were diluted to 1:100 in fresh LB and incubated for 2 hours, at which time the antibiotics were added and the sampling initiated. **b** | Growth and mortality of *E. coli* K12 MG1655 in saline. Overnight cultures of bacteria were centrifuged (washed), resuspended to their initial volume, and diluted to 1:100 in 1% saline, at which time the antibiotics were added and the sampling initiated. All cultures were maintained at 37°C with shaking at ~200 revolutions per minute. Densities were estimated by diluting and plating on LB agar. CFU, colony-forming units.

Box 1 | Simple model of non-inherited resistance and antibiotic treatment

There are two compartments in this model, one in which the bacteria are proliferating at a high rate and are highly susceptible to killing by the antibiotic (unprotected, U), and one where they are growing slowly and are less sensitive to the antibiotic (protected, P) (see FIG. 1). The concentration of the antibiotic, A, and its pharmacokinetics in the two compartments are identical. The densities of bacteria that are susceptible and genetically resistant to the antibiotics are designated S_u and R_u , and S_p and R_p , respectively, for the unprotected and protected compartments. Bacteria move (change states) from U to P and from P to U at a rate of x and y per cell per hour, respectively. In our model, the changes in the concentrations of the antibiotic and densities of the component populations are represented by a set of coupled differential equations, one for each of the bacterial states.

For the pharmacodynamic relationship between the concentration of the antibiotic and the growth or death of susceptible (S_u and S_p) bacteria, we use a Hill function⁷⁷. For our numerical analysis of the properties of this model we assume that susceptible bacteria in the U compartment have a higher maximum growth rate (1 per hour) than those in the P compartment (0.01 per hour) and correspondingly greater and smaller maximum kill rates, -10 and -0.01 for S_u and S_p , respectively. We assume that the S_u and S_p populations have the same minimum inhibitory concentrations ($1 \mu\text{g ml}^{-1}$) and Hill coefficients ($\kappa = 1$). We also assume that the S_u and S_p bacterial populations are not intrinsically (genetically) resistant to the antibiotic but that the S_p population is more refractory to the drug because of its lower rate of replication and corresponding lower rate of antibiotic-induced mortality. These pharmacodynamics are illustrated in FIG. 2a. In the simulations in BOX 2, where the evolution of resistance is considered, it is assumed that the maximum rates of replication of the genetically resistant populations R_u and R_p are unaffected by the antibiotic, but are correspondingly lower than their respective susceptible populations (0.90 and 0.009, respectively). Susceptible bacteria can mutate to the genetically resistant state at random and at a rate of μ per cell per hour. For the equations, and for further information about the model and the Berkeley Madonna program used for the numerical analysis of the properties of this model, please refer to the **Supplementary information S1** (box).



the absence of antibiotics and then exposed again, they are as sensitive to the drug as the population from which they are derived^{45,48}.

The reason why the persistent subpopulation can survive antibiotic exposure seems to be the same as that of population-wide drug indifference — the bacterial cells are not undergoing active replication. Indeed, there is compelling evidence that a subset of antibiotic-refractory bacterial cells can account for the declining mortality observed in time-kill experiments of the kind described in FIG. 1a (REFS 46,48,49). For a broader theoretical consideration of how bacteria evolve the ability to generate persistent subpopulations in response to agents that kill dividing cells, readers are referred to Kussell *et al.*⁵⁰

Various biological mechanisms have been proposed to explain how a subset of an otherwise dividing population of bacteria ceases to replicate and thereby adopts the persistent phenotype. Because bacteria exit the lag phase of growth asynchronously, growing populations will often include a subset of cells that has not yet begun to replicate^{46,51}. During the course of growth, bacterial populations include cells that are in the process of repairing damaged DNA and are not dividing while these repair processes are underway⁵². Indeed, some antibiotics induce the SOS

system, which temporarily stops bacterial cell division⁵³.

Finally, persistence can also result from the inhibition of translation of antibiotic targets by variable expression of toxin-antitoxin (TA) gene modules⁵⁴. The well-characterized *hipA* mutants of *E. coli*^{55,56}, first identified by Moyed and Bertrand, seem to generate high frequencies of persistent cells by this mechanism. More recently, *E. coli* cells that overexpress **RelE**, the toxin component of a TA module, were also shown to produce large numbers of persisters as a result of toxin-induced growth arrest⁵⁴. TA modules are a common feature of microbial genomes and could represent a general mechanism for the generation of persistent populations.

Tolerance

As noted above, the word ‘tolerance’ has been used to describe phenomena that we propose should be called persistence. Part of the reason for this semantic suggestion is that the word tolerance has been used for a different phenomenon in which cells respond to an otherwise bactericidal antibiotic as though it were bacteriostatic. In the presence of the antibiotic, tolerant bacteria cease growing; however, they do survive and can initiate replication once the

antibiotic is removed⁵⁷. Based on *in vitro* estimates of their minimum inhibitory concentrations (MICs) to a given drug, tolerant bacteria can seem as sensitive to the drug as susceptible bacteria. Although tolerance has been observed for a number of bacterial species (for example, *S. pneumoniae*⁵⁸ and *Staphylococcus aureus*⁵⁹), the phenomenon seems to be restricted to the response of bacteria to antibiotics that affect cell-wall synthesis, including the β -lactam (for example, ampicillin) and glycopeptide (for example, vancomycin) classes of antibiotic. Tolerance, which certainly should be considered in the design of antibiotic treatment regimes, is an inherited rather than a non-inherited form of antibiotic resistance; tolerant cells are genetically different to the non-tolerant bacteria from which they are derived^{60–63}.

Biofilms

Although it is convenient for both mathematical modellers and experimentalists to assume bacterial populations are composed of individual cells that exhibit a planktonic lifestyle, this view of microbial life does not accurately reflect the reality of the majority of their existence in the environment beyond well-shaken flasks and bubbled chemostats⁶⁴. In addition to growing as micro-colonies, bacteria colonizing the tissues of infected hosts also form aggregates known as biofilms. This form of bacterial growth contains genetically sensitive cells that are refractory to most, if not all, antibiotics^{64–66}. At least three reasons have been postulated and, at least partially, shown to contribute to this form of non-inherited antibiotic resistance^{56,65,66}.

First, it has been proposed that bacterial cells embedded in the polysaccharide matrices that constitute biofilms are less accessible to diffusing antibiotics (when compared with planktonic bacteria)⁶⁷. The second reason is a form of drug indifference. Because of nutrient and other limitations, many bacterial cells within a biofilm do not metabolize and replicate sufficiently for the antibiotics to function effectively^{29,68}. Third, and currently the least-supported hypothesis, is that bacteria within biofilms differentiate to states that are refractory to antibiotics for reasons other than existing in the stationary phase of growth or being deprived of nutrients⁶⁹.

A model of non-inherited resistance

To demonstrate the different ways non-inherited resistance can contribute to the microbiological outcome of antibiotic treatment, we use a simple, heuristic (rather than specific or precise) mathematical model of the worst-case scenario, where the host

defences are not operative and infection is controlled only by the antibiotic (see BOX 1 for details). In this model, part of the infecting population of bacteria is replicating at a high rate and is highly susceptible to killing by the antibiotic (unprotected), and another part of the population is replicating at a low rate and is relatively refractory to the antibiotic (protected). The protected subpopulation could be a contiguous, non-dividing, persistent subpopulation of an otherwise growing population; it could have a different physical structure to the more rapidly dividing cells as a consequence of its residence in a biofilm; or the protected subpopulation could be physically separated from the dividing population and, because of local environmental conditions, have restricted growth characteristics and therefore be refractory to the antibiotic. In this model, bacteria can either physically move between, or physiologically change between, these protected and unprotected states (or sites).

The changes in the concentration of the antibiotic (pharmacokinetics) and the consequence of having a protected population with equal rates of transition between the unprotected and protected compartments are shown in FIG. 2b. During the course of treatment, the concentration of the antibiotic oscillates above and below the MIC of the susceptible bacteria. When the concentration of the drug is above the MIC the bacterial density decreases, and when it is below the MIC the bacterial density increases. In the absence of input of bacteria from the protected compartment, the decrease in cell density exceeds the increase and the infection is cleared with three doses of the antibiotic. If, at the start of treatment, there is an established population of protected cells, the infection is not cleared by the antibiotic. The density of bacteria in the protected compartment remains approximately constant because there is little or no replication within this compartment, and the rate of flow in from the unprotected site is equal to the rate of the flow out. As a consequence of the transition to and from the protected compartment, bacteria in the unprotected compartment are also not cleared but rather recover from their initial decline and their densities continue to increase and decrease (for averages of between 1.6×10^3 and 1.8×10^4 bacteria ml^{-1} between days 2 and 14 for simulations with initial and sustained protected site densities of 10^5 and 10^6 ml^{-1} , respectively (FIG. 2b)).

Although the bacteria in the unprotected site are not completely cleared when the initial density of cells in the protected site is

low (10^3 cells) (FIG. 2c), the number of bacteria is probably too low to cause symptoms (on average 14 cells ml^{-1} between days 2 and 14), and would be predicted to be cleared in the presence of an effective host response. If dormancy can be broken, which we simulate by increasing the rate of transition from the protected to the unprotected state ($y > x$), the protected population would decrease owing to antibiotic-mediated mortality in the rapidly dividing population (FIG. 2d). The initial boost in the size of the susceptible population in the unprotected site immediately after this transition is due to the influx of susceptible cells to the site. Finally, as is shown in BOX 2, non-inherited antibiotic resistance can promote the ascent of inherited resistance. Although the intensity of selection for inherited resistance is low in the protected compartment, this site continues to serve as a reservoir for resistant mutants to enter the unprotected site (or state) where the degree of selection for resistance is high.

Conclusions and recommendations

For individual patients it is difficult to ascertain the contribution of non-inherited resistance to the duration and success of antibiotic treatment. Arguably, because of the plethora of host factors contributing to the outcome of infections, this is also the case

for inherited resistance. However, *in vitro* and experimental animal studies point to a potentially important role for non-inherited resistance in the microbiological course and success of antibiotic treatment. In addition, as detailed above, there are also theoretical arguments supporting a role for non-inherited antibiotic resistance in the outcome of antibiotic therapy. More specifically, non-inherited resistance can: prolong the time before an infecting population of bacteria is cleared from the host; preclude the clearance of an infection by antibiotics alone; and promote the generation and subsequent predominance of inherited (acquired) resistance in treated hosts (BOX 2). How commonly these clinical outcomes of non-inherited antibiotic resistance are realized remains to be seen. Phrased another way, non-inherited resistance poses largely unanswered questions that have direct implications for the successful design and execution of antibiotic therapy regimes, questions that we believe can and should be addressed.

To geneticists and other reductionist biologists, non-inherited resistance of the various forms discussed here — drug indifference, persistence and biofilms — are recognized and appreciated phenomena⁷⁰, but, similar to other kinds of physiological variation, these properties are also considered inconvenient to

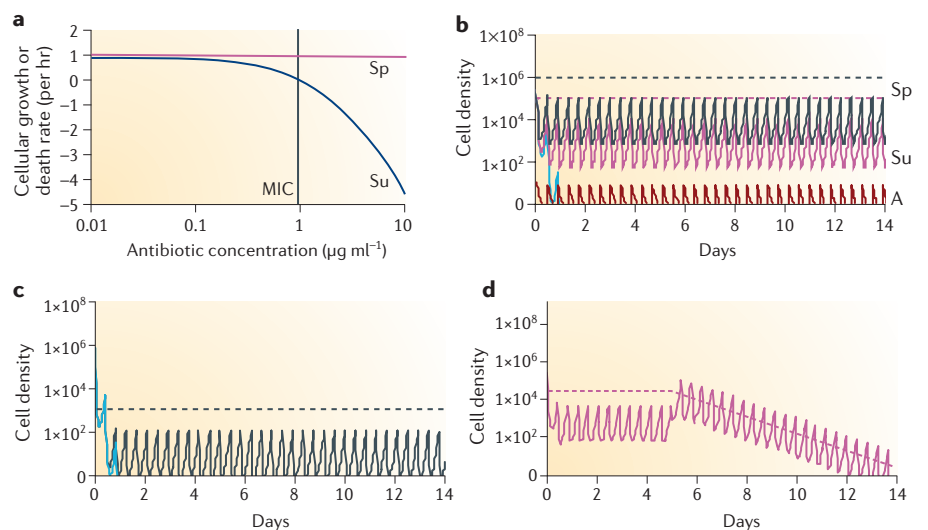


Figure 2 | The effect of non-inherited resistance on the course of antibiotic treatment (model simulation results). **a** | Changes in the growth or death rate of susceptible bacteria in the unprotected and protected compartments, Su and Sp (respectively), as a function of the concentration of the antibiotic (pharmacodynamics). **b** | Changes in the concentration of the antibiotic, A (pharmacokinetics) and densities of Su and Sp (solid and broken lines, respectively). In this simulation and that in (c) the initial density of susceptible bacteria (Su) is 10^6 and the rates of transition between U and P are equal: $x = y = 10^{-3}$. The solid blue line is the change in Su in the absence of a protected subpopulation. The black and pink lines are the changes in densities of Su and Sp with initial Sp subpopulations of 10^6 and 10^5 , respectively. **c** | Changes in the densities of Su and Sp in the absence of a protected population, (blue line) and with an initial Sp = 10^3 (black dashed line). **d** | Changes in the densities of Su with changes in the rate of transition from P to U from $y = 0.001$ to $y = 0.05$ at day 5.

deal with and are sometimes overlooked in the relevant studies. This is especially so for investigations with experimental animals, which are tedious, time-consuming, ethically unappealing and expensive inconveniences, and particularly so when compared with addressing research questions with *in vitro* studies or mathematical models. Unfortunately, these convenient tools are not sufficient; *in vivo* experiments with laboratory animals are needed not only to explore and understand the contribution of non-inherited resistance to the course of antibiotic treatment, but also for the development of agents and design of treatment protocols to counter its effects.

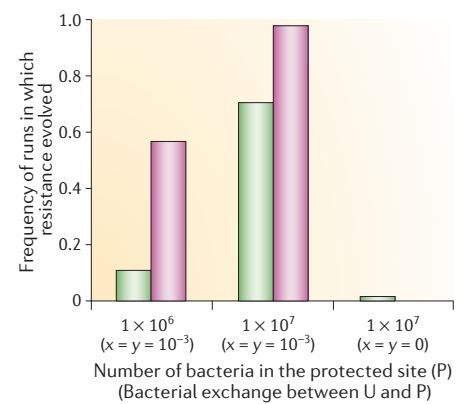
However, in combination with animal models of infection and treatment⁷¹, theoretical and *in vitro* pharmacodynamic and pharmacokinetic studies^{72,73} provide much of the crucial information required for the rational (as opposed to purely empirical) design of antibiotic-treatment protocols. These animal model treatment experiments are usually short term and commonly performed with immunocompromised (neutropenic) animals. Consequently, they offer little or no direct information about the contributions of non-inherited resistance to the course of antibiotic therapy. Laboratory animal infection and treatment experiments of the type performed by Harry Eagle, Walsh McDermott and others need to be revisited.

The crucial aspect of these experiments is to delay the administration of treatment until the infection is established in the host and begins to become symptomatic, as would be the case for a real infection in a treated patient. Although these types of laboratory animal experiment are difficult and expensive to conduct, we believe that with contemporary technology they could be more informative than they have been in the past. Imaging procedures, using fluorescent and radio labelling⁷⁴, make it possible not only to track bacteria in a treated mammal non-invasively, but also to monitor changes in their metabolic state and, with different labels assigned to the susceptible and resistant populations, to follow the changes in the frequency of antibiotic resistance: an RCA (resistance competition assay) protocol³⁶. In these experiments with established infections, it will be particularly important to monitor changes in the density and locations of susceptible bacterial populations that are not responding to the antibiotic, to observe the nature and relative magnitudes of these persistent and indifferent subpopulations, and to measure the rates of flow and/or changes in state between these different antibiotic-responsive sites or states.

Box 2 | Promotion of inherited (acquired) resistance

If bacterial mutants that are genetically resistant to the administered antibiotic are not present before the onset of treatment, and the antibiotic therapy regime is effective in rapidly reducing the number of susceptible bacteria, mathematical models suggest that antibiotic-resistant mutants are unlikely to be generated during the course of treatment⁷⁸. This phenomenon can also be observed with the model described in this article (BOX 1) by allowing for the production of mutations by a Monte Carlo protocol, in which they are generated at a rate proportional to the maximum growth rate and the total number of cells in the protected and unprotected site (see [Supplementary information S1](#) (box) for details).

Using this version of the model, which includes mutation as a stochastic process, we first consider the antibiotic treatment protocol used to generate the simulations outlined in FIG. 2, and assume a mutation rate of 10^{-8} per cell per division, a total of 10^7 bacteria in the unprotected compartment, and no exchange with a protected population (that is, only susceptible bacteria). Under these conditions, resistance arose in only 2 out of 100 runs (see Figure). On first consideration, it might seem that if there was a protected compartment increasing the total number of bacteria and extending the duration of the infection, genetically resistant mutants would arise more frequently than if there was no protected compartment. Although this would be true if the treatment continued indefinitely and the bacteria were not cleared, the presence of a protected compartment for short periods of treatment would only have a modest effect on the likelihood of resistance arising in the unprotected compartment. For example, if it is assumed that there are 10^6 bacteria in both the unprotected and protected sites, the frequency of simulations where resistance arises in the unprotected compartment would be 11% (compared with 2% in the absence of a protected compartment; see Figure). Increasing the number of bacteria in the protected site (to 10^7 bacteria) or increasing the rate of turnover and the intensity of selection in the protected compartment (pink bars) considerably augments the likelihood of resistance evolving during the two weeks of treatment. Increasing the maximum rate of replication in the protected site increases the number of generations of bacterial growth, and increasing the maximum kill rate in the protected site increases the intensity of selection. As such, there is an escalation in the likelihood that resistant mutants will make their way to the unprotected site. The figure illustrates the frequency of runs in which resistant mutants arise during 2 weeks of treatment. In all runs, the mutation rate to generate resistance was 10^{-8} per cell per division. One hundred independent simulation runs were made for each set of parameters. In the simulation results shown in green, the pharmacodynamic, pharmacokinetic and other parameters are identical to those detailed in FIG. 2. In the simulation results shown in pink, the maximum replication rate and maximum kill rate of sensitive bacteria in the protected site were 10-fold higher (0.1 hr^{-1} rather than 0.01 hr^{-1}). All other parameters are the same as those used in FIG. 2.



Of course, we also believe that mathematical and computer simulation models will be useful in evaluating the effects of non-inherited resistance on the outcome of antibiotic treatment and for the design of treatment protocols to overcome these effects. Unlike the model used here, which only considers the pharmacokinetics and pharmacodynamics of the antibiotic and bacteria, more advanced models will also have to consider the host's contribution to the generation of non-inherited antibiotic resistant states and the role of host immunity in the control and clearance of infection. Although we look forward to developing and analysing these more realistic models, at this juncture we do not believe that there is enough information to construct them or obtain realistic

estimates of their parameters. Quantitative animal model infection and treatment experiments of the types described above will be required to obtain this information.

This need to better understand the role of non-inherited resistance in antibiotic treatment has been appreciated for some time by investigators studying and designing chemotherapy protocols for the treatment of long-term (rather than acute) bacterial infections, such as tuberculosis. It is clear, even without the support of mathematical models, that drugs that can overcome bacterial latency and therefore restore antibiotic susceptibility to persistent and drug-indifferent bacteria will be the key to successful therapy for persistent infections. The 'TB Drug Accelerator Research' programme,

recently announced by the **Bill and Melinda Gates Foundation**, and its focus on agents and procedures to deal with bacterial latency (P. Small, personal communication) is a very tangible illustration of this interest and concern. In addition to reducing the efficacy of therapy in the absence of inherited resistance, as illustrated in BOX 2, it is possible that this latency can also promote the ascent of genetically resistant mutants, and therefore contribute to treatment problems associated with acquired (inherited) resistance in tuberculosis patients⁷⁵. We believe that a similar effort should be given to understanding the nature and consequences of all forms of non-inherited antibiotic resistance for the treatment of acute and persistent bacterial infections, and the development of measures to counter the effects of these phenomena. In the more distant future, we might be able to treat bacterial infections by specifically stimulating host defences to minimize their over-response to bacteria and their expressed products⁷⁶. For now and the near future, however, we will have to rely on antibiotics to control the proliferation of the infecting bacteria and, therefore, it is imperative that methods are developed to thwart resistance to these agents, be it inherited or otherwise.

Bruce R. Levin and Daniel E. Rozen are at the Department of Biology, Emory University, Atlanta, Georgia 30307, USA.
Correspondence to B.R.L.
e-mail: blevin@emory.edu
doi:10.1038/nrmicro1445

- Rubin, R. J. *et al.* The economic impact of *Staphylococcus aureus* infection in New York city hospitals. *Emerg. Infect. Dis.* **5**, 9–17 (1999).
- Lipsitch, M. The rise and fall of antimicrobial resistance. *Trends Microbiol.* **9**, 438–444 (2001).
- Levy, S. B. & Marshall, B. Antibacterial resistance worldwide: causes, challenges and responses. *Nature Med.* **10**, S122–S129 (2004).
- Fuller, J. D. & Low, D. E. A review of *Streptococcus pneumoniae* infection treatment failures associated with fluoroquinolone resistance. *Clin. Infect. Dis.* **41**, 118–121 (2005).
- Cosgrove, S. E. The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay and health care costs. *Clin. Infect. Dis.* **42**, S82–S89 (2006).
- Andriole, V. T. The quinolones: past, present and future. *Clin. Infect. Dis.* **40**, S425–S431 (2005).
- Monnet, D. L. in *The Global Threat of Antibiotic Resistance: Exploring Roads towards Concerted Action* (Dag Hammarskjöld Foundation, Uppsala Sweden, 1994).
- Lomovskaya, O. *et al.* Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrob. Agents Chemother.* **45**, 105–116 (2001).
- Boehr, D. D. *et al.* Broad-spectrum peptide inhibitors of aminoglycoside antibiotic resistance enzymes. *Chem. Biol.* **10**, 189–196 (2003).
- Mullin, S., Mani, N. & Grossman, T. H. Inhibition of antibiotic efflux in bacteria by the novel multidrug resistance inhibitors bicridar (VX-710) and timcodar (VX-853). *Antimicrob. Agents Chemother.* **48**, 4171–4176 (2004).
- Moloughney, J. G., Thomas, J. D. & Toney, J. H. Novel IMP-1 metallo- β -lactamase inhibitors can reverse meropenem resistance in *Escherichia coli* expressing IMP-1. *FEMS Microbiol. Lett.* **243**, 65–71 (2005).
- Lomovskaya, O. & Bostian, K. A. Practical applications and feasibility of efflux pump inhibitors in the clinic — a vision for applied use. *Biochem. Pharmacol.* **30**, 910–918 (2006).
- Feikin, D. R. *et al.* Mortality from invasive pneumococcal pneumonia in the era of antibiotic resistance, 1995–1997. *Am. J. Public Health* **90**, 223–229 (2000).
- Yu, V. L. *et al.* An international prospective study of pneumococcal bacteremia: correlation with *in vitro* resistance, antibiotics administered and clinical outcome. *Clin. Infect. Dis.* **37**, 230–237 (2003).
- Austrian, R. & Gold, J. Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia. *Ann. Intern. Med.* **60**, 759–776 (1964).
- Finland, M. Adventures with antibacterial drugs. *Clin. Pharmacol. Ther.* **13**, 469–511 (1972).
- Lynch, J. P. & Zhanel, G. G. Escalation of antimicrobial resistance among *Streptococcus pneumoniae*: implications for therapy. *Semin. Respir. Crit. Care Med.* **26**, 575–616 (2005).
- Sorensen, T. I., Nielson, G., Anderson, P. & Teasdale, T. Genetic and environmental influences on premature death in adult adoptees. *N. Engl. J. Med.* **318**, 727–732 (1988).
- Spitznagel, J. K. in *Mechanisms of Microbial Diseases* (eds Schaechter, M., Medhoff, M. G. & Eisenstein, B. J.) 90–114 (Williams and Wilkins, Baltimore USA, 1993).
- Zeigler, H. K. in *Mechanisms of Microbial Diseases* (eds Schaechter, M., Medhoff, M. G. & Eisenstein, B. J.) 114–153 (Williams and Wilkins, Baltimore USA, 1993).
- Levin, B. R. & Antia, R. Why we don't get sick: the within-host population dynamics of bacterial infections. *Science* **292**, 1112–1115 (2001).
- Janeway, C. A. & Travers, P. *Immunobiology: The Immune System in Health and Disease* (Current Biology Limited, London, 1996).
- Lee, S. W., Foley, E. J. & Epstein, J. A. Mode of action of penicillin I. Bacterial growth and penicillin activity — *Staphylococcus aureus* FDA. *J. Bacteriol.* **48**, 393–399 (1944).
- McDermott, W. Microbial persistence. *Yale J. Biol. Med.* **30**, 257–291 (1958).
- Paramasivan, C. N., Sulochana, S., Kubendiran, G., Venkatesan, P. & Mitchison, D. A. Bactericidal action of gatifloxacin, rifampin, and isoniazid on logarithmic- and stationary-phase cultures of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **49**, 627–631 (2005).
- Herbert, D. *et al.* Bactericidal action of ofloxacin, sulbactam-ampicillin, rifampin, and isoniazid on logarithmic- and stationary-phase cultures of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **40**, 2296–2299 (1996).
- Tuomanen, E., Cozens, R., Tosch, W., Zak, O. & Tomasz, A. The rate of killing of *Escherichia coli* by β -lactam antibiotics is strictly proportional to the rate of bacterial growth. *J. Gen. Microbiol.* **132**, 1297–1304 (1986).
- Zeiler, H. J. & Voigt, W. H. Efficacy of Ciprofloxacin in stationary phase bacteria *in vivo*. *Am. J. Med.* **82**, 87–90 (1987).
- Anderl, J. N., Zahller, J., Roe, F. & Stewart, P. S. Role of nutrient limitation and stationary-phase existence in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* **47**, 1251–1256 (2003).
- Eagle, H. Experimental approach to the problem of treatment failure with penicillin. *Am. J. Med.* **13**, 389–399 (1952).
- Eagle, H., Fleischman, R. & Musselman, A. D. The bactericidal action of penicillin *in vivo*: the participation of the host, and the slow recovery of the surviving organisms. *Ann. Intern. Med.* **33**, 544–571 (1950).
- Eagle, H. The effect of the size of the inoculum and the age of the infection on the curative dose of penicillin in experimental infections with streptococci, pneumococci, and *Treponema pallidum*. *J. Exp. Med.* **90**, 595–607 (1949).
- Darnell, J. E., Pesch, B. B. & Glaser, R. J. Effect of penicillin on group A streptococci *in vivo* in the absence of leucocytes. *J. Clin. Invest.* **34**, 1237–1241 (1955).
- Wood, W. B. & Smith, M. R. An experimental analysis of the curative action of penicillin in acute bacterial infections. 1. The relationship of bacterial growth rates to the antimicrobial effect of penicillin. *J. Exp. Med.* **103**, 487–498 (1956).
- Smith, M. R. & Wood, W. B. An experimental analysis of the curative action of penicillin in acute bacterial infections. 3. The effect of suppuration upon the antibacterial action of the drug. *J. Exp. Med.* **103**, 509–522 (1956).
- Bull, J. J., Levin, B. R., DeRouin, T., Walker, N. & Bloch, C. A. Dynamics of success and failure in phage and antibiotic therapy in experimental infections. *BMC Microbiol.* **2**, 35 (2002).
- Negri, M. C., Lipsitch, M., Blazquez, J., Levin, B. R. & Baquero, F. Concentration-dependent selection of small phenotypic differences in TEM β -lactamase-mediated antibiotic resistance. *Antimicrob. Agents Chemother.* **44**, 2485–2491 (2000).
- Smith, H. W. & Huggins, M. B. Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *J. Gen. Microbiol.* **128**, 307–318 (1982).
- Zappala, R. *The Population Biology of Antibiotic Treatment Failure in Immune-competent Mice*. Ph. D. Dissertation, Emory Univ. (2004).
- McCune, R. M. & Tompsett, R. Fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. 1. The persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. *J. Exp. Med.* **104**, 737–762 (1956).
- Toman, K. Bacterial persistence in leprosy. *Int. J. Lepr. Other Mycobact. Dis.* **49**, 205–217 (1981).
- Musser, J. M., Gray, B. M., Schlievert, P. M. & Pichichero, M. E. *Streptococcus pyogenes* pharyngitis — characterization of strains by multilocus enzyme genotype, M-protein and T-protein serotype, and pyrogenic exotoxin gene probing. *J. Clin. Microbiol.* **30**, 600–603 (1992).
- Fitoussi, F. *et al.* Molecular DNA analysis for differentiation of persistence or relapse from recurrence in treatment failure of *Streptococcus pyogenes* pharyngitis. *Eur. J. Clin. Microbiol. Infect. Dis.* **16**, 233–237 (1997).
- Clement, S. *et al.* Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent *Staphylococcus aureus* rhinosinusitis. *J. Infect. Dis.* **192**, 1023–1028 (2005).
- Bigger, W. B. Treatment of staphylococcal infections with Dexticillin. *Lancet* **244**, 497–500 (1944).
- Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. Bacterial persistence as a phenotypic switch. *Science* **305**, 1622–1625 (2004).
- Barclay, M. L., Begg, E. J. & Chambers, S. T. Adaptive resistance following single doses of gentamicin in a dynamic *in vitro* model. *Antimicrob. Agents Chemother.* **36**, 1951–1957 (1992).
- Wiuff, C. *et al.* Phenotypic tolerance: antibiotic enrichment of non-inherited resistance in bacterial populations. *Antimicrob. Agents Chemother.* **49**, 1483–1494 (2005).
- Levin, B. R. Microbiology. Non-inherited resistance to antibiotics. *Science* **305**, 1578–1579 (2004).
- Kussell, E., Kishony, R., Balaban, N. Q. & Leibler, S. Bacterial persistence: a model of survival in changing environments. *Genetics* **169**, 1807–1814 (2005).
- Metris, A., Le Marc, Y., Elfving, A., Ballagi, A. & Baranyi, J. Modelling the variability of lag times and the first generation times of single cells of *E. coli*. *Int. J. Food Microbiol.* **100**, 13–19 (2005).
- Debbia, E. A., Roveta, S., Schito, A. M., Gualco, L. & Marchese, A. Antibiotic persistence: the role of spontaneous DNA repair response. *Microb. Drug Resist.* **7**, 335–342 (2001).
- Miller, C. *et al.* SOS response induction by β -lactams and bacterial defense against antibiotic lethality. *Science* **305**, 1629–1631 (2004).
- Keren, I., Shah, D., Spoering, A., Kaldalu, N. & Lewis, K. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J. Bacteriol.* **186**, 8172–8180 (2004).
- Moyed, H. S. & Bertrand, K. P. *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J. Bacteriol.* **155**, 768–775 (1983).
- Lewis, K. Persister cells and the riddle of biofilm survival. *Biochemistry (Moscow)* **70**, 267–274 (2005).
- Tomasz, A., Albino, A. & Zanati, E. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. *Nature* **227**, 138–140 (1970).
- Rodríguez, C. A. *et al.* Tolerance to vancomycin in pneumococci: detection with a molecular marker and assessment of clinical impact. *J. Infect. Dis.* **190**, 1481–1487 (2004).
- Rajashakraiah, K. R. *et al.* Clinical significance of tolerant strains of *Staphylococcus aureus* in patients with endocarditis. *Ann. Intern. Med.* **93**, 796–801 (1980).
- Tomasz, A. & de Vegvar, M. L. Construction of a penicillin-tolerant laboratory mutant of *Staphylococcus aureus*. *Eur. J. Clin. Microbiol.* **5**, 710–713 (1986).

61. Tuomanen, E., Durack, D. T. & Tomasz, A. Antibiotic tolerance among clinical isolates of bacteria. *Antimicrob. Agents Chemother.* **30**, 521–527 (1986).
62. Tuomanen, E. Phenotypic tolerance: the search for β -lactam antibiotics that kill nongrowing bacteria. *Rev. Infect. Dis.* **8** (Suppl. 3), 279–291 (1986).
63. Tuomanen, E. & Tomasz, A. Mechanism of phenotypic tolerance of nongrowing pneumococci to β -lactam antibiotics. *Scand. J. Infect. Dis.* **74**, S102–S112 (1990).
64. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322 (1999).
65. Lewis, K. Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* **45**, 999–1007 (2001).
66. Stewart, P. S. & Costerton, J. W. Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135–138 (2001).
67. Anderl, J. N., Franklin, M. J. & Stewart, P. S. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* **44**, 1818–1824 (2000).
68. Gilbert, P., Collier, P. J. & Brown, M. R. W. Influence of growth-rate on susceptibility to antimicrobial agents — biofilms, cell-cycle, dormancy, and stringent response. *Antimicrob. Agents Chemother.* **34**, 1865–1868 (1990).
69. Southey-Pillig, C. J., Davies, D. G. & Sauer, K. Characterization of temporal protein production in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* **187**, 8114–8126 (2005).
70. Elowitz, M. B., Levine, A. J., Siggia, E. & Swain, P. Stochastic gene expression in a single cell. *Science* **297**, 1183–1186 (2002).
71. Andes, D. & Craig, W. A. Animal model pharmacokinetics and pharmacodynamics: a critical review. *Int. J. Antimicrob. Agents* **19**, 261–268 (2002).
72. Mouton, J. W., van Ogtrop, M. L., Andes, D. & Craig, W. A. Use of pharmacodynamic indices to predict efficacy of combination therapy *in vivo*. *Antimicrob. Agents Chemother.* **43**, 2473–2478 (1999).
73. Drusano, G. L. Antimicrobial pharmacodynamics: critical interactions of 'bug and drug'. *Nature Rev. Microbiol.* **2**, 289–300 (2004).
74. Bettegowda, C. *et al.* Imaging bacterial infections with radiolabeled 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)-5-iodouracil. *Proc. Natl Acad. Sci. USA* **102**, 1145–1150 (2005).
75. Harris, K. A. Jr, Mukundan, U., Musser, J. M., Kreiswirth, B. N. & Lalitha, M. K. Genetic diversity and evidence for acquired antimicrobial resistance in *Mycobacterium tuberculosis* at a large hospital in South India. *Int. J. Infect. Dis.* **4**, 140–147 (2000).
76. Kolls, J. K. & Nelson, S. Immune modulation in the treatment of respiratory infection. *Respir. Res.* **1**, 9–11 (2000).
77. Regoes, R. R. *et al.* Pharmacodynamic functions: a multiparameter approach to the design of antibiotic treatment regimens. *Antimicrob. Agents Chemother.* **48**, 3670–3676 (2004).
78. Lipsitch, M. & Levin, B. R. The population dynamics of antimicrobial chemotherapy. *Antimicrob. Agents Chemother.* **41**, 363–373 (1997).

Acknowledgements

We wish to thank F. Baquero, K. Drlica, P. Small and C. Wiuff for comments and suggestions. This enterprise was supported by grants from the US National Institutes of Health and the British Wellcome Trust (IPRAVE Project).

Competing interests statement

The authors declare no competing financial interests.

DATABASES

The following terms in this article are linked online to:

Entrez Genome Project: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj>
Escherichia coli | *Mycobacterium tuberculosis* |
Staphylococcus aureus | *Streptococcus pneumoniae* |
Treponema pallidum
 UniProtKB: <http://ca.expasy.org/sprot>
 ReLE

FURTHER INFORMATION

Author's homepage: www.eclif.net

Bill and Melinda Gates Foundation: <http://www.gatesfoundation.org>

Access to this links box is available online.