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Lethal Mutagenesis of Bacteria

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ABSTRACT

Lethal mutagenesis, the killing of a microbial pathogen with a chemical mutagen, is a potential broad-spectrum antiviral treatment. It operates by raising the genomic mutation rate to the point that the deleterious load causes the population to decline. Its use has been limited to RNA viruses because of their high intrinsic mutation rates. Microbes with DNA genomes, which include many viruses and bacteria, have not been considered for this type of treatment because their low intrinsic mutation rates seem difficult to elevate enough to cause extinction. Surprisingly, models of lethal mutagenesis indicate that bacteria may be candidates for lethal mutagenesis. In contrast to viruses, bacteria reproduce by binary fission, and this property ensures their extinction if subjected to a mutation rate >0.69 deleterious mutations per generation. The extinction threshold is further lowered when bacteria die from environmental causes, such as washout or host clearance. In practice, mutagenesis can require many generations before extinction is achieved, allowing the bacterial population to grow to large absolute numbers before the load of deleterious mutations causes the decline. Therefore, if effective treatment requires rapid population decline, mutation rates $\gg 0.69$ may be necessary to achieve treatment success. Implications for the treatment of bacteria with mutagens, for the evolution of mutator strains in bacterial populations, and also for the evolution of mutation rate in cancer are discussed.

A high mutation rate can cause population extinction. With viruses, application of this principle is known as lethal mutagenesis (Loeb *et al.* 1999; Sierra *et al.* 2000; Pariente *et al.* 2001; Grande-Perez *et al.* 2002; Anderson *et al.* 2004; Freistadt *et al.* 2004; Graci *et al.* 2007, 2008), and the artificial elevation of mutation rate with drugs (chemical mutagens) is practiced to cure or control an infection (Smith *et al.* 2005; Chung *et al.* 2007). However, lethal mutagenesis has been attempted only with viruses that have RNA genomes, whose intrinsic mutation rates are high enough ($\sim$1 per genome per generation) that they might be pushed over an extinction threshold with only a slight mutational increase. DNA viruses have genomic mutation rates nearly two orders of magnitude lower than those of RNA viruses (Drake 1993; Drake *et al.* 1998) and are therefore not considered candidates for lethal mutagenesis.

Lethal mutagenesis has not been proposed as a control strategy for bacteria. Like viruses with DNA genomes, bacteria have low intrinsic genomic mutation rates (0.003 mutations per genome per replication; Drake *et al.* 1998), so bacteria would seem to be poor candidates for this type of control. However, the extinction threshold derived for viruses contains a fecundity term that is two to three orders of magnitude higher in viruses than the equivalent for bacteria, so extinction by lethal mutagenesis may be far more attainable for bacteria than for DNA viruses (Bull *et al.* 2007).

A second and perhaps more intriguing possibility is that bacteria might *evolve* a mutation rate that is high enough to cause extinction (Andre and Godelle 2006; Gerrish *et al.* 2007). The models on which that process is based do not actually invoke an extinction threshold, so the results derived here allow one to assess whether bacteria might evolve to extinction by a more standard process. Collectively, these possibilities motivate the calculation of a lethal mutagenesis threshold for bacteria, which we attempt here. We do not model the evolution of mutation rate, but simply consider how a mutation rate (perhaps externally applied) affects persistence of a large asexual bacterial population, not subject to stochastic processes.

METHODS

We implemented a continuous-time simulation of bacterial growth and death. The population consisted of N bacteria with growth rates b_i and death rates d_i, proceeding in discrete time steps of infinitesimal length δt. In each time step, a bacterium with birth rate b_i and death rate d_i had probability $b_i \delta t$ of dividing and a probability $d_i \delta t$ of dying. Dead bacteria were removed from the population. If the bacterium reproduced, it was replaced by two possibly mutated daughter cells. Muta-

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tion was a Poisson process; after replication each cell received k new deleterious mutations, where k is a Poisson-distributed random variable with mean U_d.

Mutations affected the birth rate of bacteria multiplicatively. A bacterium with n mutations had birth rate $b(1 - s)^n$. Back mutations were not allowed. For all simulation results presented in this work, we set all death rates to $d_i = d = 0.1$. The absolute value of d is arbitrary and in essence determines the length of unit time. All results depend only on the relative magnitude of the quantity b as compared to d. As length for the infinitesimal time step, we used $\delta t = 0.01$. This value is also arbitrary, but must satisfy $\delta t \ll d$ for reliable numerical simulation.

We determined the critical value of the mutation rate U_d using a divide-and-conquer algorithm. Starting with a mutation-rate interval (U_{min}, U_{max}) large enough to contain the critical U_d with certainty, the population was seeded with N_0 bacteria and grown while experiencing mutation rate $U_{new} = (U_{min} + U_{max})/2$. If the population reached size N_{max}, we considered the population as not destined for extinction and set $U_{max} = U_{new}$. Otherwise, if the population went extinct without reaching N_{max}, we set $U_{min} = U_{new}$. The process was then repeated with a newly seeded population, until $(U_{max} - U_{min})/U_{max} < 5\%$. Finally, the critical mutation rate was set to the average of the final mutation-rate-interval boundaries, $(U_{max} + U_{min})/2$.

RESULTS

Background: A review of results for viruses provides the foundation for the present study. For a wild-type, asexual virus with a progeny number of b per infected cell and a genomewide, deleterious mutation rate of U_d (the average number of deleterious mutations per genome, per replication, regardless of the sizes of the deleterious effects), extinction is ensured in the largest of populations if

$$be^{-U_d} < 1 \qquad (1)$$

(Bull *et al.* 2007). This model is the simplest possible, assuming that the distribution of deleterious mutations per genome is Poisson, that mutation is coupled with replication, and that mutations arise only in the progeny genomes as each is copied from the parent template. The value of b is the number of progeny from a wild-type viral infection that would go on to infect new cells except for any loss due to mutation. Therefore, b is an effective burst size, not the actual number of virions produced per infected cell, if some virus is lost to host clearance. Remarkably, U_d is simply the deleterious mutation rate, regardless of the magnitude of effect of those mutations.

This threshold can be rationalized in two ways, and understanding these arguments will facilitate understanding the bacterial case. One way recognizes that e^{-U_d} is the mean relative fitness of an equilibrium asexual population with deleterious mutation rate U_d (Kimura and Maruyama 1966), so the left-hand side of (1) is simply the mean absolute fitness at equilibrium (average progeny number). When population fitness declines to the point that the average infection produces less than one successful offspring, the population size must decline. In this interpretation, the effective burst size declines as mutations accumulate: the value b applies to the wild type, so genotypes with lower fitness have less than b successful offspring.

Alternatively, e^{-U_d} represents the fraction of genomes that are free of new deleterious mutations. The left-hand side of (1) thus represents the average number of progeny from an infected cell that are identical to the parent, and when this quantity is less than one, the mutation-free class declines. If all mutations are lethal, then it is obvious that (1) ensures extinction. But extinction also occurs even when the deleterious mutations are not lethal. When the mutation-free class (0 class) is present in the population, (1) ensures its loss. Once the 0 class has disappeared, (1) ensures the loss of the 1-mutation class, and so on, until the number of deleterious mutations per genome is so large that the burst size has fallen to the point that the population cannot maintain itself. Again, the assumption is that the effective burst size declines with genome mutation load.

There are typically two gross components of viral fitness, burst size and generation time. Changes in burst size (of the wild type) obviously affect the threshold. Changes in generation time do not affect this simple threshold—because mutation is coupled to replication instead of time, with one episode of mutation per generation. A more realistic model might have two mutational processes, one increasing with time and the other tied to replication events. Such a model has not been considered because there is no practical way of increasing the per-time mutation rate of the virus without also increasing the mutation rate of the host cell. Agents that would damage nonreplicating viral genomes would also likely damage the cellular genomes, an undesirable form of treatment. However, drugs that interfere with viral replication can (and do) selectively increase the viral mutation rate during replication. Thus the high mutation rate is assumed to be coupled with replication instead of time.

The simplest model for bacteria: The parallel model for bacteria assumes that bacteria do not age and that they reproduce by fission, with each daughter cell acquiring new mutations at random. This symmetry of mutation rates between the two daughters is appropriate for semiconservative replication (Tannenbaum *et al.* 2004). Such a population would be expanding indefinitely, with no death in the absence of mutation. This population growth is unrealistic, but the process is easy to comprehend, so it serves as a useful starting point. The counterpart to (1) is

$$2e^{-U_d} < 1, \tag{2a}$$

and the mutation rate at the extinction threshold is

$$U_d = \ln(2) = 0.69. \tag{2b}$$

This threshold is the point at which a bacterial division produces exactly one mutation-free daughter cell on average.

This first model establishes one basic difference between the viral and the bacterial case that persists even in realistic extensions of the model: there is a low upper limit on bacterial mutation rates (0.69) that arises from the symmetry in bacterial reproduction. Viral thresholds can be high because burst sizes can be high (*e.g.*, 1000), but bacterial division sets the effective burst size at 2. Some qualifications apply, as discussed below, but this limit is a robust feature of bacterial lethal mutagenesis.

The model assumes that mutation happens at replication, independent of the time between replications. Perhaps surprisingly, the only phenotypes that affect the extinction threshold are survival and the ability to divide. Consequently, not all mutations that have deleterious effects are counted in U_d in this model (a restriction that will change below when the model's realism is improved). In particular, mutations affecting generation time do not affect the threshold. Thus, a mutation that reduced bacterial growth rate would be selected against in a growing population, but that mutation would not affect the lethal mutagenesis threshold in this model—it would not affect whether a bacterial division produces less than one mutation-free offspring.

Incorporating bacterial birth and death: The previous model captured the most fundamental property of lethal mutation for an organism that undergoes binary fission when reproducing. This section offers a more realistic extension of that model. Perhaps the most serious limitations of the previous model are that it assumes an absence of extrinsic bacterial death, and it neglects generation time (hence U_d omitted mutations that slow generation time). More typically, bacterial populations will die from extrinsic causes, and death will operate per unit time. These considerations motivate a model based on actual time rather than generation time.

Let N be the number of wild-type bacteria whose birth rate is b and death rate is d, both operating per unit time. Death can be from many causes, such as killing by phages or an immune response, or merely washout from the environment. We assume that mutation coincides with birth/reproduction, which is tantamount to assuming that mutations arise during genome replication and are not expressed until segregated into a daughter cell. The model relies on the fact that reproduction can be represented as a parent that dies after giving birth to two offspring, each of which may have independently acquired mutations (an assumption consistent with the semiconservative nature of DNA replication). The number of wild-type bacteria N increases by one if both offspring are mutation free, it decreases by one if both offspring contain one or more deleterious mutations, and it remains unchanged if one offspring is mutation free and the other is not. Let p be the probability that an offspring acquires one or more deleterious mutations. The probability that both offspring are mutation free is then $(1-p)^2$, and the probability that neither is mutation free is p^2. Therefore, N satisfies

$$\frac{dN}{dt} = \big[(1-p)^2 b - p^2 b - d\big]N = \big[(1-2p)b - d\big]N. \tag{3}$$

Under the Poisson model of mutation, the probability p is related to the deleterious mutation rate U_d via $p = 1 - e^{-U_d}$. Inserting this expression into (3), we find

$$\frac{dN}{dt} = \big[(2e^{-U_d} - 1)b - d\big]N. \tag{4}$$

Similar equations have been studied in the semiconservative version of the quasi-species model (Tannenbaum *et al.* 2004; Tannenbaum and Shakhnovich 2005), with the main difference that our Equation 4 lacks a normalization term that keeps the population size constant. Thus our model specifically describes increases and decreases in the population whereas the other does not.

Formally, Equation 4 describes the fate of the mutation-free population of individuals (the 0 class) all experiencing the same U_d, b, and d. The 0 class will die out if the quantity inside the brackets is <0, hence if

$$e^{-U_d} < \frac{1}{2}\left(1 + \frac{d}{b}\right) \tag{5a}$$

or

$$U_d > \ln(2) - \ln\left(1 + \frac{d}{b}\right). \tag{5b}$$

The mutation rate at the lethal threshold, U_d^*, is found by replacing the inequality with equality. If all deleterious mutations are lethal, then this condition obviously prescribes the loss of all individuals. If individuals with one or more mutations survive, then (5b) prescribes loss of the 0 class, at which point all individuals will have one or more deleterious mutations. Those mutations will lower the birth rate below b (hence also increasing generation time), increase the death rate above d, or cause some combination of changes to birth and death. Note that a death rate of zero recovers the result (2) from above.

With loss of the 0 class, the values of b and d will have changed, and individuals with different mutations will usually have different parameter values. (We designate the new, individual-specific birth and death rates as b_i, d_i.) An equation of the same form as (4) will thus apply to them, but there will be a different equation for each set

of (b_i, d_i). The question is whether the former threshold U_d^* is sufficient for mutations to continue accumulating with the new b_i and d_i. For deleterious changes that reduce b, that increase d, or both, the threshold U_d^* from (5b) now exceeds that required for mutation accumulation, so lethal mutagenesis will in fact accelerate. There is, however, a small zone in parameter space for which formerly deleterious mutations not only become beneficial in the presence of mutagenesis but also can push the individual outside the realm of lethal mutagenesis. This possibility exists only for mutation rates near the extinction threshold U_d^*, and the mutations must have the pleiotropic effect of reducing death rate more than they reduce birth rate scaled by $2e^{-U_d}$. Those mutations are thus treated as beneficial rather than deleterious and are not part of U_d (see below).

In this new model, the lethal mutagenesis threshold remains bounded by 0.69, but several effects can push the threshold to much lower rates. Indeed, there are environments in which only a trivial increase in mutation rate is required for extinction. From (5b), a sufficiently high bacterial death rate can poise the population ever so close to extinction before any increase in mutation rate is applied. Intuitively, this must be true, because raising the death rate above the birth rate will ensure extinction even without mutation. Deleterious mutation is merely one of several factors that can contribute to population decline.

Another difference from the first model is that a broader spectrum of mutations is considered deleterious in this second model. U_d now includes mutations reducing birth rate (hence increasing generation time) as well as those increasing death rate. These results have parallels with viral lethal mutagenesis. Components of birth and death can both be accommodated in the viral model through their effects on burst size: b is the number of virions released from an infected cell that also go on to infect new cells, so the viral b will decline if virion death rate increases or burst size declines. A generation-time effect has not yet been considered for the viral case, but it could be accommodated in the same fashion as here, by including a constant viral death rate.

Serial transfer: The special case of serial transfer is of interest, as some of the easiest experiments with bacteria use this protocol. With serial transfer, a period of bacterial growth for a fixed interval (T time units) is followed by an aliquot of the completed culture being transferred to a new culture of the same volume. If the final culture is diluted to a concentration of $1/D$ in the new culture, then D will be the amplification that occurs from the beginning to the end of a single culture. Derivation of the extinction threshold follows from the observation that an exponentially growing population will die out if it cannot increase its density by D between transfers, or

$$\frac{N(T)}{N_0} < D, \tag{6a}$$

where N_0 is the initial number of bacteria at time $t = 0$. Assuming exponential growth, extinction requires

$$e^{rT} < D, \tag{6b}$$

where $r = [(2e^{-U_d} - 1)b - d]$ is the growth rate of the bacterial culture, obtained from the right-hand side of (4). Thus

$$r < \frac{\ln(D)}{T}. \tag{6c}$$

The extinction threshold is then easily modified from (5b) as

$$U_d > \ln(2) - \ln\left(1 + \frac{d + \ln(D)/T}{b}\right), \tag{6d}$$

again bounded by 0.69.

For most experimental designs, the death rate can be treated as zero. However, some explanation of the birth rate is required. At least at the start of such experiments, the duration T is long enough that bacterial densities increase substantially before transfer, so the birth rate declines between the start and the end of a single culture. Which value of b should be used? To an approximation, the value that should be used in (6d) is the average birth rate that operates in a culture whose density has increased by D from the lowest possible starting density (perhaps the transfer of a single individual). The value of b under these conditions will typically be larger than when the culture starts at higher density, because saturation will occur earlier when the starting density is higher. The justification for using this magnitude of b is that, as extinction is approached, the bacterial density at the start of a culture will ultimately decline to the low-density limit.

In reality, the process is complicated by two factors beyond those considered in this model. First, a bacterial culture that saturates should be modeled as logistic growth (Yano *et al.* 1998). Logistic growth entails two parameters, a birth rate and a carrying capacity, both of which would be affected by deleterious mutations. Second, when bacterial density saturates and reaches stationary phase, the bacteria transferred to a new culture experience a lag before initiating growth, so they do not obey exponential growth (Lenski *et al.* 1994). A lag phase will not apply if bacterial densities do not saturate, so the model's neglect of a lag phase is not a problem in protocols with sufficiently rapid transfers. Despite these inaccuracies, the thresholds calculated here should be suitable approximations for capturing effects within an order of magnitude.

Extinction in practice requires mutation rates much higher than predicted: The thresholds for lethal mutagenesis calculated above are deterministic and allow an infinity of time for population extinction. Their appeal is in their generality, in that the details of the sizes of

deleterious effects, epistasis, and other types of genetic details are irrelevant. All that is required is the deleterious mutation rate. Yet it takes many, many generations to approach the equilibrium mutation load when the effects of those mutations are weak (Johnson 1999a). Although the first mutations will start accumulating immediately upon increase in the mutation rate, the mean fitness of the population will be slow to decline when mutational effects are small, because it takes an accumulation of many mutations to produce a major impact on fitness.

From a practical perspective, lethal mutagenesis is feasible only if the population can be controlled quickly. Thus, if the application of an elevated mutation rate allows the population to increase 1000-fold before the growth rate becomes negative, treatment may be pointless. To gain insight to the magnitude of population growth under different forms of lethal mutagenesis, we offer some numerical examples. The magnitude of the deleterious mutation effect s does indeed have a profound impact on both the time at which the population starts declining and the increase in population size before decline (Figure 1A, for a starting population size of 1000). With large effects ($s = 0.5$), the population barely grows before the decline sets in, and extinction is achieved at ~100 generations. As deleterious effects drop and approach 0, the increase in population size and time to extinction go up dramatically. At $s = 0.04$, there is an ultimate 100-fold increase in population size following mutagenesis and nearly 500 generations to extinction. Dynamics of genomes with combinations of these mutational effects would be intermediate to those shown. All illustrations have assumed a death rate of $d = 0.1$; elevating the death rate further would also hasten extinction. For comparison, loss of the zero class is nearly independent of s (Figure 1B), so the delayed impact on population size is due to the time to accumulate an adequate number of mutations per individual.

A related perspective is obtained by considering the lowest mutation rate required to prevent the population from growing beyond a size limit before the decline starts. When mutations are deleterious but not lethal, this size-limiting mutation rate will always exceed the rate for strict lethal mutagenesis (Figure 2). As expected, the size-limiting mutation rate is higher with smaller deleterious effects, but the magnitude of this effect can be quite large: the extinction threshold U_d^* can be elevated 10-fold for reasonable values of s. Perhaps surprisingly, increasing the size limit has only a modest effect on the critical mutation rate (Figure 2B). The general effects illustrated in Figures 1 and 2 apply to viruses as well as to bacteria.

Beneficial mutations impede extinction: The preceding models omitted any consideration of neutral or beneficial mutations. Neutral changes do not affect the process, provided that they remain neutral even as other mutations accumulate. The neglect of beneficial mutations is problematic, however. Mutations that increase birth rate, reduce intrinsic death rates, or even reduce the deleterious effects of other mutations all have an advantage under many conditions and will be favored. Importantly, these types of mutations may also offset extinction.

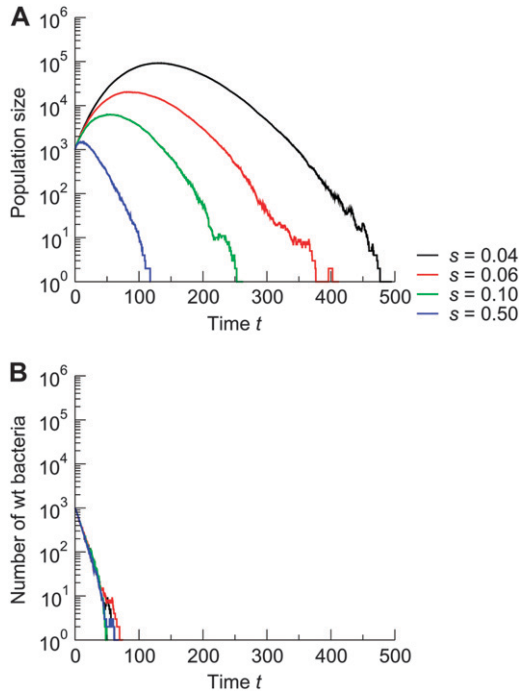

Figure 1.—Total population size and number of mutation-free bacteria under mutagenesis as a function of time, in numerical simulations of replicating and mutating bacteria. Parameters are $b = 0.2$, $d = 0.1$, $N_0 = 1000$, $U_d = 0.7$. Deleterious mutations affect birth rate only. A bacterium with k deleterious mutations has fitness $b(1 - s)^k$. (A) Total bacterial population size as a function of time. (B) Number of wild-type bacteria as a function of time.

Modeling the evolution of beneficial mutations is complicated, and the process is specific to many details of the model. A general descriptor of population evolution for arbitrary mutation rates and fitness effects is given by the system of linear differential equations

$$\frac{dN_i}{dt} = \sum_j T_{ij} N_j, \tag{7}$$

where N_i is the number of bacteria of type i, and the matrix

$$T_{ij} = 2b_j Q_{jj} - \delta_{ij}(b_i + d_i) \tag{8}$$

describes the processes of birth, death, and mutation of bacteria. The constants b_i and d_i are the birth and death rates of type i, and Q_{ij} is the mutation probability from j to i. The symbol δ_{ij} is 1 if $i = j$ and 0 otherwise. The population is guaranteed not to go extinct if at least one of the eigenvalues of T_{ij} has a positive real part. Unfortunately, there are no generalities about the role of

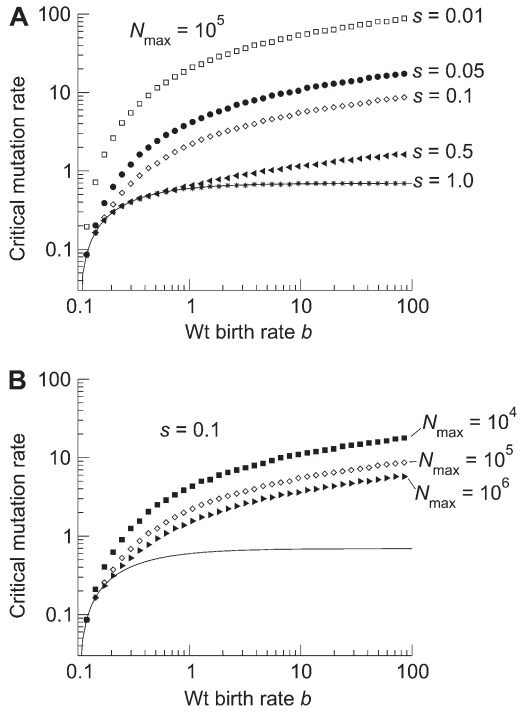

FIGURE 2.—Critical mutation rate as a function of wild-type birth rate b. A population that can grow to the size N_{max} is considered to be below the critical mutation rate. A population that instead goes extinct before reaching N_{max} is above the critical mutation rate. Points represent simulation results, and the solid line in each panel corresponds to the strict lethal threshold from Equation 5b. Parameters are $d = 0.1$ and $N_0 = 1000$. Deleterious mutations affect birth rate only. A bacterium with k deleterious mutations has fitness $b(1 - s)^k$.

mutation in population persistence that follow from these equations without further simplifications.

One simplification is to suppose that there are a modest number of beneficial mutations increasing birth rate and/or decreasing death rate and whose effects are robust to genetic background. To obtain the maximum effect on the extinction threshold for these types of beneficial mutations, one would assume that they were present before any elevation in the mutation rate. This biology could be modeled as merely a higher starting birth rate (b^*) and a lower starting death rate (d^*). The extinction threshold is then easily recalculated from (5b) as $U_d > \ln(2) - \ln(1 + d^*/b^*)$. This approach provides a direct comparison of the extinction threshold before and after the evolution of beneficial mutations, but only when the effects on birth and death rates are known. Mutations that reduce the mutation rate (such as by lowering the intracellular concentration of a chemical mutagen or improving polymerase fidelity) will also be beneficial but will not affect the threshold; they will merely decrease the chance that the bacterium reaches the threshold.

Not all beneficial effects can be treated so simply, however. The fraction of all mutations that are beneficial is suspected of increasing as mean fitness declines (POON and OTTO 2000), which means that the value of U_d decreases as deleterious mutations accumulate. This dynamic behavior of U_d could raise the mutation rate needed to cause extinction, but the details would be specific to the fitness landscape. (The threshold may or may not be affected by this dynamical behavior of U_d, because mutation accumulation may accelerate with mutation load enough to offset the effect.) The picture is further complicated by the fact that deleterious mutations will be individually rare in the population. Thus, many beneficial mutations will be "conditionally" beneficial, compensatory for the specific deleterious mutations already present in a genome, but not beneficial for the deleterious mutations present in other genomes. Understanding adaptive evolution during lethal mutagenesis is thus in need of further work.

DISCUSSION

This article has presented simple models for extinction of a bacterial population by lethal mutagenesis—elevating the mutation rate to a level that deleterious mutations ultimately cause the population to die out. Lethal mutagenesis has been proposed as a mechanism to cure viral infections but has not been proposed for treating bacterial infections. The reason for this taxonomic bias may be simply that some viruses have RNA genomes, whose intrinsic mutation rates are already so high (DRAKE 1993; DRAKE *et al.* 1998) that it seems feasible to boost their mutation rates "over the top." Lethal mutagenesis has not been entertained for DNA genomes, and bacteria as well as many viruses have DNA genomes. However, bacteria differ from DNA viruses by having an intrinsically high propensity for extinction by mutagenesis: bacterial extinction is ensured if the deleterious mutation rate exceeds 0.69 per replication, whereas the threshold for DNA viruses is often far higher.

A gross estimate of bacterial genomic mutation rates from comparative data is 0.003 per replication (DRAKE *et al.* 1998); a lower bound for the rate of nonlethal, deleterious mutations in *Escherichia coli* is 0.0002 (KIBOTA and LYNCH 1996). These values are far below the basic threshold of 0.69. However, the threshold for extinction can be depressed below 0.69 by several factors that depend on the environment. Whereas extinction is ensured at high mutation rates, extinction may also be caused by low mutation rates in environments that are marginally conducive to bacterial survival.

By contrast, two factors may boost the extinction threshold well above 0.69, so that extinction is even harder to achieve. One is the practical matter of achieving extinction within a reasonable time and without excessive population expansion. If most deleterious mutations have small effects, the mutation rate needed to enforce extinction within practical constraints may increase severalfold. Second, increases in the extinction threshold

also result from beneficial mutations. The magnitude of this latter effect can be calculated for simple cases but requires knowing the effect of beneficial mutations on birth and death rates.

The rate of approach to the deleterious mutation equilibrium is hastened if deleterious mutations are lethal—the impact of lethals is realized in one generation. Fully 40% of 70 randomly generated point mutations evaluated singly in the RNA virus, vesicular stomatitis virus (VSV) were genomic lethals (Sanjuan *et al.* 2004). If this fraction of lethals was maintained during mutagenesis, at least 40% of the approach to equilibrium would happen immediately. This estimate of lethals may be unrealistic for bacteria, however. For cellular proteins, the fraction of mutations destroying function is typically 30%, but ranges from 0.03 to 0.6 (Shakhani *et al.* 1997; Daugherty *et al.* 2000; Guo *et al.* 2004; Drummond *et al.* 2005). A mutation that destroys protein function would be lethal to a genome only if the gene was essential. As the fraction of genes in *E. coli* estimated to be strict lethals is only ~14% (Gerdes *et al.* 2003), it can be inferred that the fraction of mutations that are lethal in bacteria is substantially lower than the estimate for VSV. The lethal fraction estimated for *E. coli* was based on growth in rich media (*e.g.*, only 16% of amino acid biosynthesis genes were essential in this environment), which may be appropriate for lab propagation but probably not for a heterogeneous, natural environment.

A high mutation rate may in principle be imposed externally, as with the use of ribavirin to treat infections caused by some RNA viruses (Smith *et al.* 2005; Chung *et al.* 2007). As yet, there are no drugs for bacteria that specifically increase the point mutation rate, but some antibacterial drugs work partly by DNA damage. Quinolones induce the formation of chemical radicals that damage DNA and other cellular components (Kohanski *et al.* 2007). DNA damage is critical to the effect of these drugs, because cells lacking in DNA repair exhibit enhanced susceptibility. This DNA damage does not necessarily lead to point mutations, however, and to the extent our models apply, most of the "mutations" would be lethal. Some other antibiotics work by blocking DNA replication (*e.g.*, nalidixic and oxolinic acids). Here the effect is not mutagenic, but the existence of drugs specific for bacterial DNA replication points toward the possibility of drugs to inhibit replication fidelity in bacteria. Nonetheless, the imposition of lethal mutagenesis does not yet appear to be a practical approach for treating bacterial infections, for lack of appropriate drugs.

If lethal mutagenesis is not yet feasible for bacteria, there are nonetheless strong motivations for studying it in experimental settings. Foremost, testing extinction thresholds should lead to basic insights about mutation and fitness. Furthermore, a process akin to lethal mutagenesis can operate without external forcing, as described next.

An excessively high mutation rate may also *evolve* through natural selection. Whenever a population can experience rare beneficial mutations, those mutations will always be more likely to arise in genotypes with atypically high mutation rates ("mutator" strains) than in genotypes with lower mutation rates. In the absence of recombination, a mutator genotype with the beneficial mutation may ascend and even ultimately sweep through the population to fixation (a process known as "hitchhiking"), elevating the population mutation rate far above the optimum (Chao *et al.* 1983; Sniegowski 1997; Sniegowski *et al.* 1997; Taddei *et al.* 1997; Shaver *et al.* 2002; Gerrish *et al.* 2007). In the short term, population fitness must increase from this process, or the mutator genotype would not be able to replace all nonmutator genotypes. In the long term, fitness may decline if the mutation load eventually exceeds the gain from the beneficial effect. The load may not approach equilibrium until long after the sweep of the beneficial mutation (Johnson 1999a,b; Andre and Godelle 2006; Gerrish *et al.* 2007), although the fraction of mutations that are lethal imposes a limit on how long the equilibrium load may be delayed. In theory, it is possible for the population to evolve itself to extinction from iterations of this process (Andre and Godelle 2006; Gerrish *et al.* 2007). Our model does not address the evolution of mutation rate, but it provides the first quantitative formulation of the minimal mutation rate that is needed for evolution to extinction.

Experimental transfers of bacteria fail to support the evolution of reduced fitness through mutators, but the evolved mutation rates may be too low to expect a profound effect. The most useful studies for providing quantitative insights are those characterizing the long-term *E. coli* B lines of Lenski, propagated by daily serial transfer in minimal media with a 100× dilution fraction (Sniegowski *et al.* 1997). Of 12 replicate lines, each carried through 20,000 generations, 8 lines showed no evidence of mutators at high frequency, whereas in 3 lines mutator phenotypes arose early and persisted for thousands of generations to the end. The estimated elevation in mutation rate depended on the phenotype studied, but seemed to lie between 10-fold and 100-fold (50-fold in one article). The estimate of 0.003 for the baseline, initial mutation rate (Drake *et al.* 1998) would leave the per-generation, genomic mutation rate between 0.03 and 0.3, significantly below the extinction threshold of 0.69 (direct calculation of the genomic rate is not possible from the data). In the extreme case that all mutations were deleterious, the predicted fitness at equilibrium from the load would lie between 0.97 and 0.74. Fitnesses of the mutator lines were not statistically distinguishable from fitnesses of the nonmutator lines, a result that is perhaps unsurprising given the relatively small expected deleterious load and stochastic differences between lines in the ascent of beneficial mutations.

Although the models in this work have been framed around bacteria, they are also relevant to cancer. Furthermore, consideration of cancer leads to a better understanding of the bacterial case. Some cancer treatments are indeed mutagenic, such as X rays, but the killing of cancer cells with X rays is not a meaningful application of the models, because the goal with X rays is to kill cancer cells outright rather than to continuously mutate them and cause a gradual buildup of deleterious mutations. The relevance of our model to cancer is the evolution of high mutation rates and the consequent possibility of extinction. There is now overwhelming evidence that many cancers exhibit a mutator genotype/phenotype. The types of mutations have even been divided into two distinct classes, chromosomal instabilities and microsatellite instabilities, reflecting differences in segmental mutations *vs.* point mutations (Charames and Bapat 2003). Although the mutator phenotype doubtless evolves because it hastens the occurrence of mutations benefitting cellular growth, there must also be a load from mutations deleterious to cancer growth. Is it possible that a cancer could evolve a high enough mutation rate that it went "extinct" from its deleterious load? Not likely.

One of the substantial differences between the bacterial evolutionary model and cancer involves population turnover. In the bacterial model, a mutator strain hitchhikes to fixation, replacing all ancestors and competitors with lower mutation rates. In a cancer, at least a solid tumor, this type of replacement does not occur. The cancer grows out, but there is not a clean exchange of new cells for older cells. Thus, cells with lower mutation rates are not lost; they just lack the same number of beneficial mutations as those with higher mutation rates and thus are not increasing as fast. A cancer, therefore, presents a living fossil record of its history of normal cells and those with progressively higher and higher division rates (and thus progressively greater abundances). Even if some cell lines die out from their mutations, their progenitors remain alive and well to continue the uncontrolled growth. An additional complication of cancer is that the deleterious mutation rate is profoundly difficult to ascertain. A rogue cell does not require many of its genes, and the genomes of cancer cells are so poorly behaved, with deletions and duplications of large segments, that it is difficult to even apply the concept of a constant deleterious rate.

The empirical evidence from mutator strains in bacteria and cancer highlights the need for more theoretical work to accommodate the effect of beneficial mutations into the extinction threshold. Beneficial mutations can clearly have a large effect on the mutation threshold, but we do not understand the processes by which they would accumulate in an asexual population in which most genotypes are unique. A more sinister problem arising from our neglect of beneficial mutations is that the application of mutagenesis could possibly lead to a "superbug" that not only tolerated the higher mutation rate but also accumulated several beneficial mutations that improved it over the initial strain. One question that might be answered empirically and would go toward addressing the general issue of the effect of beneficial mutations is whether mutator strains achieve higher ultimate fitness than their progenitors with low mutation rates.

It is not surprising that the empirical side of bacterial lethal mutagenesis is poorly understood at this point, as viral lethal mutagenesis remains poorly understood despite considerable empirical work (Sierra *et al.* 2000; Pariente *et al.* 2001; Anderson *et al.* 2004; Vignuzzi *et al.* 2005; Graci *et al.* 2007). Ribavirin is the drug most commonly considered as a candidate for causing lethal mutagenesis of RNA viruses. However, ribavirin is suspected of inhibiting viral replication by at least five mechanisms, only one of which is mutagenesis (Graci and Cameron 2006). Although ribavirin does indeed elevate the mutation rate of several viruses, it is not known whether this elevation is necessary or sufficient to cause extinction. An additional complication is that exposure of virus to ribavirin commonly selects resistance mutants with high polymerase fidelity, thus reducing the error rate of replication [demonstrated for poliovirus (Pfeiffer and Kirkegaard 2003)]. Other types of resistance mutations and beneficial mutations no doubt occur as well. Yet another possible form of response to an extrinsically imposed mutation elevation is the evolution of robustness to mutation (Van Nimwegen *et al.* 1999; Wilke 2001; Wilke *et al.* 2001; Foster *et al.* 2006; Codoñer *et al.* 2006; Sanjuán *et al.* 2007). Overall, the work on lethal mutagenesis of viruses reveals that the realm of possible outcomes is diverse, and the complexities are not trivial to discriminate.

Other approaches to survival limits imposed by mutation rates have been considered. Perhaps the most renowned mutational cause of extinction is Muller's ratchet, in which stochastic processes lead to a systematic increase in mutation load (Muller 1964; Haigh 1978; Maynard Smith 1978; Gessler 1995; Rouzine *et al.* 2003, 2008). The ratchet will operate at lower mutation rates than those given by our extinction threshold, although the decay in fitness from the ratchet is also slower. Furthermore, the rate of the ratchet depends on the sizes of deleterious effects. The strict extinction thresholds calculated here do not depend on sizes of mutational effects, but the practical thresholds do. The deleterious effects of mutation may also be calculated from the perspective of thermodynamic protein stability and loss of function (Guo *et al.* 2004; Bloom *et al.* 2005, 2007; Wilke *et al.* 2005; Zeldovich *et al.* 2007). In particular, Zeldovich *et al.* (2007) calculated a universal extinction threshold of six mutations per essential portion of the genome per replication. This threshold should apply to all life forms, as long as mutations arise

independently of replication, *e.g.*, through ionizing radiation. If mutations arise exclusively during replication, the mathematical formalism of Zeldovich *et al.* (2007) does not apply without modification, and the extinction threshold will likely have a different value. It is an open question for future experimental work whether the extinction threshold of mutagenesis via error-prone replication and that via ionizing radiation are experimentally distinguishable.

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