ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2008, p. 1209–1214 Vol. 52, No. 4
0066-4804/08/$08.00+0 doi:10.1128/AAC.01152-07


# MINIREVIEW

# A Practical Guide to Measuring Mutation Rates in Antibiotic Resistance▽

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Bacteria become resistant to antibacterial agents by three main mechanisms: acquisition of complete resistance genes or gene complexes via plasmids and other transposable elements (12, 16, 21, 26, 30), recombination of DNA from other bacteria into the genome by transformation (6), and spontaneous mutational events in the chromosome and accessory DNA (14). Horizontal gene transfer in bacteria has been reviewed by Thomas and Nielsen (31). This minireview will concentrate on the study of chromosomal mutations that confer resistance. Mutational events are assumed to be stochastic, so that the rate of beneficial mutation does not occur at a higher frequency than those that are neutral or disadvantageous and that mutations are not directed. For bacterial cells, there is a finite probability that a mutation conferring the resistant phenotype will occur, and unless a revertant mutation occurs, all of the progeny of such a cell will be resistant also. An important review by Rosche and Foster which critically analyzes mutation rate determination methods lays the foundation of this minireview (29). The terms and abbreviations used here are defined in Table 1.

## MUTATION RATE OR MUTATION FREQUENCY

A mutation rate is an estimation of the probability of a mutation occurring per cell division and corresponds to the probability of a mutation occurring in the lifetime of a bacterial cell. A mutation frequency is simply the proportion of mutant bacteria present in a culture. These terms are often used interchangeably, causing confusion. The relationship between mutation frequency and the rate at which mutations occur is uncertain. If a mutation arises early in the culture period, then a large number of mutant progeny occur and this would be represented by a high frequency. This phenomenon is known as a "jackpot culture" and was first described in 1943 by Luria and Delbrück during their seminal set of experiments investigating the mutation of *Escherichia coli* from bacteriophage T1 sensitivity to resistance (19). Understanding of this phenomenon was the crucial evidence indicating the role of mutation in phage resistance and underpins all of the work on mutation that followed.

## FLUCTUATION TEST OF LURIA AND DELBRÜCK

Luria and Delbrück demonstrated that bacteriophage-resistant mutant colonies arise from a sensitive culture of *E. coli* if bacteriophage T1 is present in excess (19). Resistant colonies appeared from sensitive cultures, i.e., in which there was clearing, within 12 to 16 h. These bacteria were resistant to bacteriophage T1 but sensitive to other viruses capable of causing lysis in that strain of *E. coli*. Luria and Delbrück showed that reversion to sensitivity was a rare event and that, in a growing culture, the proportion of resistant bacteria increased with time. They argued that if the presence of the phage was needed to trigger the change to resistance, then the distribution of mutant colonies should demonstrate a Poisson distribution. The high variance in the numbers of mutants in the culture, however, led Luria and Delbrück to conclude that resistant mutants were present in the culture before bacteriophage exposure and that the bacteriophage resistance mutation arose independently. The Luria-Delbrück distribution is different from the Poisson distribution in that its variance is greater than 1.

Luria and Delbrück assumed that for a bacterium there was a small fixed chance that a resistance-conferring mutation could occur per unit of time if the bacteria are "in an identical state." The number of mutated cells in a culture depends on how early the mutation occurred during the growth of the bacterial population. If mutation occurs early in the culture, the number of mutated cells will be higher than if it occurs later. Measurement of the mutation rate, rather than frequency, should be the standard in antibiotic research. Although the protocols and calculation methods are more complex, they are not as inaccessible as it might appear.

## DETERMINATION OF MUTATION RATE

Broadly, there are two methods for determination of the mutation rate: mutation accumulation and fluctuation analysis. Mutant accumulation methods have the advantage that they are very accurate, but they are complicated and time-consuming to perform because the culture is sampled at multiple time points. The methodology depends on growing bacteria exponentially until probability dictates that a mutant will be present. If the assumption is made that the growth rates of wild-type and mutant bacteria are the same, then the proportion of mutants will increase linearly with time. Furthermore, if the number of mutants and the total number of bacterial cells are known at each time point, then the mutation rate ($\mu$) can be calculated from the slope of the line describing the rela-

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▽ Published ahead of print on 4 February 2008.

TABLE 1. Terms and abbreviations used in this minireview

| Term | Definition |
|---|---|
| $m$ | No. of mutational events/culture |
| $\mu$ | Mutation rate |
| $r$ | Observed no. of mutants |
| $x$ | Median no. of mutants |
| $C$ | No. of cultures |
| $p_0$ | Proportion of cultures without mutants |
| $N_0$ | Initial no. of cells |
| $N_t$ | No. of cells at time $t$ |
| $f$ | Mutant frequency |
| $n$ | No. of generations |

tionship between the number of mutants against the generation number. The mutation rate can be determined by using the equation $\mu = [(r_2/N_2) - (r_1/N_1)] \times \ln (N_2/N_1) = (f_1 - f_2) \times \ln (N_2/N_1)$, where $r_1$ is the observed number of mutants at time point 1, $r_2$ is the observed number of mutants at the next time point, and $N_1$ and $N_2$ are the numbers of cells at time points 1 and 2, respectively, while $f_1$ and $f_2$ are the mutant frequencies at points 1 and 2.

For this method to be accurate, a very large difference in the total cell number is required between $N_1$ (the number of cells at the first time point) and $N_2$ (the number of cells at the second time point). Serial dilutions would make this easier to perform, but this introduces sampling errors. If available, continuous culture would be an alternative but this would allow the selection of waves of bacteria, each better suited than the generation before to take over the culture (25). Moreover, many studies have shown that the acquisition of a mutation providing resistance is associated with a significant fitness deficit, which invalidates one of the basic premises of the mutant accumulation method, as less fit mutants will accumulate at a different rate than the parent (3). For this reason and for greater simplicity, fluctuation methods are more commonly used, and this minireview will concentrate on describing various applications of this approach.

## FLUCTUATION ANALYSIS IN ANTIBIOTIC RESEARCH: GENERAL PRINCIPLES

Fluctuation analysis involves estimating the mutation rate from the distribution of mutants in a number of parallel cultures. This method was pioneered by Luria and Delbrück (19). Briefly, an initial inoculum of cells (with a known cell volume) from a growing culture is added to a broth and incubated in the absence of selective pressure. The bacterial cells are concentrated and screened for antibiotic-resistant mutant cells by plating the whole cell population onto solid medium containing a suitable concentration of the test antibiotic, usually at two to four times the MIC. It is assumed that this will inhibit the growth of susceptible cells, leaving only resistant mutants. A plate count is performed on a portion of the culture to determine the number of viable cells in the cell deposit. The method of Miles and Misra can be used to determine viable cell numbers. This method involves the spotting of replicate 20-μl drops of broth onto a plate and counting of the colonies that grow within that spot. This reduces the bacterial cells that are lost by spreading (23). Luria and Delbrück suggested two methods for estimating the overall mutation rate of the population: the $p_0$ method, which is based on the proportion of cultures in which there are no mutants observed, and the method of the mean, which relies on the determination of the mean number of mutants. Both methods assume a Poisson distribution with a mean and variance equal to the product of the probability of a mutation and the number of bacteria. All of the methods described in this minireview use an estimate of the number of mutational events (not the number of mutants), $m$, to determine the mutation rate and have a Luria-Delbrück distribution (19). Parameter $m$ will be influenced by the amount of growth and the mutation rate ($\mu$). The estimated value of $m$ can be divided by the total number of cells to give the mutation rate.

## DESIGNING A MUTATION RATE EXPERIMENT

Choice of selective antibiotic. Ideally, mutation rates should be calculated by using an antibiotic to which resistance arises via a mutation at a single base pair for the reasons noted above. This situation rarely arises, and consequently, pragmatic compromises must be made. Manipulating the culture volume growth conditions and durations enables these methods to be adapted to answer a wide range of questions in antibiotic research.

The choice of the selecting agent depends on the purpose of the experiment. Antibiotics which are most suitable for mutation rate methods are those to which resistance arises as a result of point mutations in chromosomal genes, including the aminoglycosides, quinolones, rifampin, pyrazinamide, and isoniazid (10). If one wants to measure the rate of resistance to a particular antibiotic, then the nature of the drug-bacterium interaction will dictate how the parameters vary and "ranging" experiments may be required. Not all of the colonies growing on the selective plate will contain the same mutation. Thus, a mutation rate calculated by including confirmed mutations in a single target gene will be lower than a phenotypic mutation rate due to the presence of multiple target genes and nonheritable changes. In antibiotic research, it is usual that lethal selection for preexisting mutations, as in the case of the experiment of Luria and Delbrück, is being tested, and this is different from the nonlethal selection used by Cairns et al. in their "directed-mutation" experiments with Lac, which allowed mutants arising postplating to grow (5).

**Parameters.** For each mutation rate experiment, there are three main parameters which must be considered, i.e., the expected number of mutational events, the number of cultures to be examined, and the size of the initial inoculum.

If the $p_0$ method is to be used, $m$ should be between 0.3 and 2.3 mutational events per culture. If $m$ is less than 0.3, then none of the mutation rate methods are reliable. When $m$ is greater than 2.3, the Luria and Delbrück method of the mean can be used to estimate the mutation rate (19). Methods of the mean or median described below have constraints on the number of mutants per culture if the results are to be valid, and these ranges are shown in Table 2.

The number of mutational events present in the culture depends on the mutation rate itself and the amount of growth. Growth conditions will vary between bacterial species. For example, culture aliquots of *Streptococcus pneumoniae* cannot be incubated for extended periods. This is due to the activity of

TABLE 2. Appropriateness of different methods for different values of *m*

| Method | Value of *m* (no. of mutational events/culture) |
|---|---|
| $p_0$ method | $0.3 \leq m \leq 2.3$ |
| Method of the mean | Any |
| Lea and Coulson method of the median | $1.5 \leq m \leq 15$ |
| Drake formula | $m \geq 30$ |

the cell wall autolysin, which results in a decrease in the viable cell count following extended incubation (15). The value of *m* can be manipulated by inoculating different volumes of broth onto solid medium, but this can introduce errors (see below). The choice of methods will vary with different values of *m*, and therefore the method chosen will depend on the expected value of *m*.

**Number of cultures.** The second crucial parameter is the number of parallel cultures (*C*) chosen to represent the bacterial population. Irrespective of the method used, the precision of *m* is a function of $1/\sqrt{C}$ and increases as *C* increases; if more cultures are tested, then precision is increased. Between 20 and 30 cultures are routinely included (2). For the $p_0$ method, a precision level of 20% is considered necessary to provide a suitable estimate of the number of mutational events per culture (29). Precision is the coefficient of variation, $\sigma_m/m$, multiplied by 100% and has been calculated as 0.2 (29) and is a measurement of the reproducibility of results, as opposed to accuracy.

**Size of initial inoculum.** The final parameter is the size of the initial inoculum ($N_0$). This inoculum should not contain any preexisting mutants, and thus it should be small. For example, in their *E. coli* experiments, Luria and Delbrück used an initial inoculum of between 50 and 500 bacteria (19). The smaller the initial inoculum, the longer the incubation period. This is especially important when working with slow-growing cultures, e.g., *Mycobacterium tuberculosis*. We have found that between 3,000 and 5,000 cells/ml is sufficient as the initial inoculum for *S. pneumoniae* and *M. tuberculosis*, respectively. There are other complications involved in growing small numbers of organisms. For example, many organisms monitor the density of cells via quorum sensing and only switch on virulence genes after a quorum of bacteria is present (24, 28). A small inoculum may produce a reduction in viability, resulting in greater variation in the final number of cells ($N_t$). In each parallel culture, the final cell number ($N_t$) should be the same and the value of $N_0$ should always be negligible compared to $N_t$ (a ratio of at least <1:1,000 is desirable). Variations in $N_t$ can be eliminated by using a large initial inoculum. Rosche and Foster (29) found that, in their experiments, a pragmatic compromise between the above factors was to use an initial inoculum of total cells of $m\,N_t/10^4$ (3, 9, 10). To reduce variability, the initial inoculum should consist of an even cell suspension. This is especially important when working with organisms such as *M. tuberculosis*, which tend to form cellular aggregates. To overcome this problem, the initial inoculum should be passed through a fine-needle syringe or a filter to form a single-cell suspension. Additionally, Middlebrook 7H9 broths contain Tween 80 to reduce clumping (3).

**Additional relevant considerations. (i) Volume.** In order to observe a mutation, it is necessary to have a large enough final cell number. The size of this final cell number is a function of the culture volume and the mutation rate. If the mutation rate is high, then a small broth culture can be used, and if the rate is low, then larger cultures must be used.

**(ii) Cell cycle.** Mutation rates may be influenced by the growth phase of the cell. Determinations of mutation rates are usually performed by using cells growing in exponential phase (3, 10). There are reports, however, that mutation rates in *E. coli* are elevated in stationary phase compared to exponential phase (15, 18). The initial inoculum of cells should contain cells that are in the same phase of the growth cycle in order to compare estimated rates. Therefore, a growth curve should be constructed during method optimization. To reduce the degree of variability in these experiments, all of the above parameters should be kept constant between experiments.

## ASSUMPTIONS OF FLUCTUATION ANALYSIS

Each mutation rate method relies on a set of pragmatic assumptions that are made in order to make estimations possible. (i) The probability of the mutation occurring is constant per cell lifetime. (ii) The probability of this mutation occurring does not vary between growth phases. (iii) There is no cell death. (iv) Revertants occur at a negligible rate. (v) Mutation occurs only during cell division and results in only one mutant. (vi) The growth rates of mutants and nonmutants are the same. (vii) Initial cell numbers are negligible compared to the final cell numbers. (viii) All mutants are detected, and no mutants occur after selection is imposed. However, these assumptions may not be true in all situations. Mutation rates of the same organism that are obtained by using the same selection tool and estimated via different methods can be very different.

## DEVIATIONS FROM THE ASSUMPTIONS

Fitness of mutants. As noted above, mutation rate calculation methods assume that there is no physiological impairment of mutants with respect to their susceptible parents. If mutants do not grow as efficiently as their parents, they may not be detected and this may affect the calculated mutation rate. There are examples in which mutations responsible for resistance occur at no or low cost. For example, the *rpsL* Lys42Arg mutation, which confers resistance to streptomycin in *Salmonella enterica* serovar Typhimurium, incurs no measurable cost. In contrast, the Lys42Thr and Lys42Asn mutations associated with resistance incur a heavy fitness burden (4). For example, the *parC* and *gyrA* mutations, conferring fluoroquinolone resistance, incur no or low cost in *S. pneumoniae* (11). The extent of a fitness deficit is dependent on the nature of the mutation, as demonstrated by *M. tuberculosis*, where there is a relationship between the rates at which various resistant mutants are found in clinical practice and the initial fitness deficit of the mutant strain (3, 8, 22).

**Completeness of detection.** It is possible that not all mutations are detected. For example, mutations that occur late in the culture may not give rise to colonies and these mutants will not be counted. This phenomenon is known as phenotypic lag. Importantly, it is also possible that mutations may occur after

selection has been imposed; i.e., mutants may arise on antibiotic-containing solid medium. In order to overcome these issues, some preliminary ranging experiments could be preformed which would ensure that the correct initial inoculum and the correct final plating volume are used. Colonies should be counted as earlier as possible to minimize the number of postplating mutants that could occur.

**Other factors.** There are a number of other factors that complicate the calculation of mutation rates. For example, mutation rates are not constant in a population of cells. They can vary depending on the antibiotic concentration (13) and the availability of the carbon source (1).

## PLATING A PORTION OF THE CULTURE

It is assumed that all mutants are detected. Plating a portion of the culture can introduce an error in the estimation of $m$. Some of the methods used to determine the rate of mutation have been derived to take sampling into account. Ma et al. (20) and Jones (14) have altered their fluctuation analysis method to show that it is possible to plate an aliquot of the culture volume when there is a large final inoculum. It is also possible to plate a portion of a large culture rather than using multiple small cultures, and Crane et al. have proposed a modified fluctuation assay for the estimation of mutation rates where small increases in the mutation rate are expected (6).

## VARATIONS ON THE LURIA-DELBRÜCK METHOD FOR MUTATION RATE ESTIMATION

No satisfactory solution of the Luria and Delbrück distribution has been found that effectively describes the distribution numerically. Therefore, extensive attempts have been made to improve the accuracy of the estimates (2, 15). The practical effect of this is that mutation rates estimated via different methods cannot be compared.

## CALCULATION METHODS

**The $p_0$ method.** The $p_0$ method is the simplest method to calculate and is the one originally described by Luria and Delbrück in their seminal paper (19). It is most suitable when the number of mutational events in a culture is low. This method has successfully been used to estimate mutation rates in *M. tuberculosis* (3) and *S. pneumoniae* (10).

The proportion of cultures without mutants ($p_0$) is the zero term of the Poisson distribution given by the equation $p_0 = e^{-m}$. This method should only be used if the proportion of cultures without mutants is between 0.1 and 0.7, i.e., the number of mutational events per culture is between 0.3 and 2.3. The formula can be rearranged to give the number of mutational events as follows: $m = -\ln p_0$.

Multiple parallel cultures are performed and scored as positive if they yield a resistant mutant, i.e., show growth. When the proportion of mutants detected is known, then the actual value of $m$ can be calculated. There is no need to enumerate the colonies, and this simplifies the process. It should be noted that the precision of $m$ varies depending on the value of $p_0$. Compared with other methods, the $p_0$ method requires more cultures for the same level of precision when $m \geq 1.2$. As cultures are scored as either positive or negative for growth, mutations that affect the growth rate of the progeny cells have less effect in the $p_0$ method than on other methods. A clone that does not give rise to a colony would add to the proportion of cultures without mutants erroneously. Conditions of growth and culture volume need to be chosen so that the proportion of resistant cultures is in the appropriate range. The $p_0$ estimator method is very sensitive to phenotypic lag, postplating mutations, and decreased plating efficiency, as these will increase the value of $p_0$. Some of the progeny of each mutant will be lost if the plate efficiency is less than 100%. This will be the normal situation in most culture systems; thus, cultures with few mutants may be counted as cultures with no mutants.

**Methods using the mean.** The mean estimator methods use the observation that when a population is large enough there will be an extra $\mu N_t$ mutants after each generation as each of the cells in the final population may undergo mutation. The probability of this occurring is determined by the mutation rate ($\mu$). Therefore, the extra number of mutants will be a product of these two terms. The time period after the point when the bacterial population of all cultures has reached the required size when this may occur is $1/\mu$ and is known as the Luria-Delbrück period. The mean methods should not be used if there is no Luria-Delbrück period, i.e., if $N_t < 1/\mu$. Methods that use the mean are disproportionally inflated by jackpot cultures and are not recommended. They can be made more accurate by removing data points caused by jackpot cultures, but this makes the approach somewhat arbitrary, with data being removed by the investigator. Methods using the median are more accurate and will be discussed in more detail below.

**Lea and Coulson method of the median.** Lea and Coulson (17) attempted to develop a method with better precision than the method of the mean. The function $m$ is calculated from the equation $(x/m) - \ln m = 1.24$.

The method assumes that if the median number of mutants is large enough, then most mutations occur early enough to be detected. From a practical point of view, a greater number of selective plates (approximately 5 to 10) are needed for this method to give an adequate precision level. An additional drawback to the increased number of plates required is that median methods should not be used if more than half of the plates are devoid of mutants. It is used when all or most of the cultures give rise to mutant colonies, and it has been quoted as the method of choice (excluding maximum-likelihood methods) if $m$ is between 1.5 and 15 and if the median number of mutational events in a culture is between 2.5 and 60. The main drawback of the method is that it is sensitive to any variation in the assumptions, e.g., phenotypic lag and altered growth rate of progeny, described previously, which results in reduced precision.

**Drake formula using the median.** The Drake formula using the median provides an easy option to make an estimate of the mutation rate from frequency data, given by the equation $\mu = f/\ln(N_t\mu)$, where $f$ is the final mutation frequency (7). By using the median final mutation frequency and not the mean final mutation frequency, the impact of jackpot mutations is reduced. It can be used when the number of mutational events per culture is high, i.e., $\geq 30$. This method has been used to estimate rates of mutation of *S. pneumoniae* to fluoroquin-

olone resistance and of *Mycobacterium fortuitum* to fluoroquinolone, macrolide, and aminoglycoside resistance (9).

**Jones median estimator.** Jones calculated the hypothetical dilutions required so that half of the selective plates in a putative experiment had mutant colonies (14). Under these circumstances, other median methods cannot be used. The Jones method has the advantage that it relies on the observed number of mutant colonies to estimate $m$ by an explicit equation. Jones (14) verified this method against the Lea and Coulson method of the median, by using computer simulations, for values of $m$ between 1.5 and 10 and showed that it is more efficient than the Lea and Coulson method of the median. Crane et al. modified the method so that it can be used to give more precise mutation rate measurements. In this method, a portion of larger-volumes cultures is plated rather than the whole of smaller-volume cultures (6); this allows more mutants to accumulate. We have used this method to estimate the rate of the *rpoB* mutation, which confers rifampin resistance (3).

## CHOOSING A METHOD

There may be a number of differences between methods, but they all use similar functions. Since the pivotal experiments by Luria and Delbrück in the last century, novel methods for the calculation of the Luria-Delbrück distribution have made the estimation of mutation rates more accurate and easy to perform. The most useful methods are the $p_0$ method (2) and the Jones median estimator together with the modification of Crane et al. for partial plating (6).

Mutation rate studies have been performed with a number of organisms related to antibiotic research, including *E. coli*, *S. pneumoniae* (10), *Pseudomonas aeruginosa* (27), and *M. tuberculosis* (3). Oliver et al. used the modification by Crane et al. of the Jones estimator to show that antibiotic-resistant isolates of *P. aeruginosa* were present prior to antibiotic therapy due to the existence of hypermutable bacteria (27). As large broths were used, aliquots from these broths were taken to reduce culture-to-culture variation (27). For example, Billington et al. also used this method for experiments with *M. tuberculosis* for similar reasons (3). Mutation rate experiments with *S. pneumoniae* have been used to show that mutations in the *gyrA* gene occur at a lower rate than *parC* mutations and that mutations in either gene predisposes to further mutation (10). The Drake method was used when ciprofloxacin was the selective antibiotic as the number of mutational events per culture was >30. However, the $p_0$ method was used with gemifloxacin as the number of mutational events was smaller (between 0.3 and 2.3).

## SUMMARY

Whichever method is chosen, the experimental factors should be optimized to improve the precision and accuracy of the estimation. It is usually necessary to perform preliminary experiments to provide estimates of the mutation rate, and as has been stated previously, it is usually helpful to determine growth curves to confirm that the bacteria are in the same growth phase when the mutation rate estimation cultures are inoculated. The growth conditions of the experiment can only be established with the knowledge of the expected mutation rate, which requires preliminary experiments to enable the researcher to develop the necessary protocol. When mutations are likely to be rare, then $m$ is, by definition, small and thus the $p_0$ method is likely to be the most useful. When $m$ is greater, then median methods are most appropriate. The choice of calculation method will depend on whether all of the cultures were positive, with a median method being chosen for situations in which all are positive and the $p_0$ when this is not the case.

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