

Measurement of Mutation to Antibiotic Resistance: Ampicillin Resistance in *Serratia marcescens*

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Abstract: The public health problem of antibiotic resistance is becoming a major threat to modern medicine. We have chosen the biological phenomenon of ampicillin resistance in the Gram-negative bacterium *Serratia marcescens* to design a laboratory exercise to illustrate this problem. Our exercise is an adaptation of the Luria-Delbrück fluctuation test, a quantitative measure of spontaneous mutation rate. We show that the mutation rate of *S. marcescens* to ampicillin resistance at 25 micrograms antibiotic per milliliter is approximately 2.3×10^{-6} mutations per cell division event. This exercise is a quantitative illustration of microbial evolution, and it is adaptable to classes of ten or more students.

Key words: *Serratia marcescens*; antibiotic resistance; Luria-Delbrück fluctuation test; spontaneous mutation

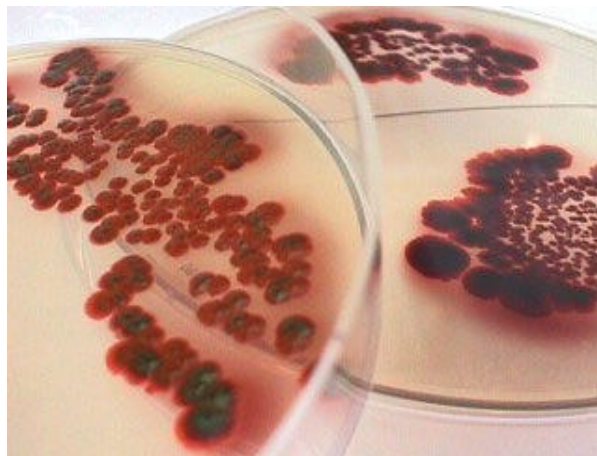
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INTRODUCTION

Antibiotics are the drugs of choice for the treatment of bacterial infections. Bacterial resistance to antibiotic therapy, however, is becoming a major threat to modern medicine. Scientists are not certain exactly how this problem arose, but current thinking seems to favor continued use of these drugs to treat infections. In this scenario, antibiotics have acted as Darwinian selective agents, killing susceptible organisms and allowing the expansion of the drug resistant variants in the population.

The biological mechanisms which lead to an antibiotic resistant phenotype are diverse, but they can be classified into three general categories (Dever & Dermody, 1991). First, bacterial enzymes can actively degrade the drug. Resistance to the beta-lactam antibiotic ampicillin (Ap), for instance, can be mediated by the enzyme beta-lactamase. Ap normally functions as an irreversible inhibitor of transpeptidase enzymes, whose function is to cross-link structural components of the bacterial cell wall. Beta-lactamase enzymes hydrolyze beta-lactam rings and render such antibiotics incapable of reacting with bacterial transpeptidase enzymes. Another example is the

aminoglycoside antibiotic kanamycin, a bacterial protein synthesis inhibitor. Enzymes conferring the kanamycin-resistant phenotype inactivate the drug by covalent attachment of a phosphate or other group.



Second, bacteria can develop mutations that render the target molecule unable to interact with the antibiotic. Streptomycin (Sm) is an aminoglycoside antibiotic effective against a wide variety of bacteria.

Spontaneous streptomycin-resistant *E. coli* can be selected under laboratory conditions (Miller 1972). Resistance to this protein synthesis inhibitor maps to the *rpsL* gene, which encodes a ribosomal protein (Hershey 1987; Davis 1987). Another example is the DNA gyrase inhibitor nalidixic acid. DNA gyrase is a supercoiling enzyme which modifies DNA topology, and it is essential for DNA replication. Nalidixic acid inhibits gyrase action by stably binding a gyrase/DNA reaction intermediate (Drlica 1987). Nalidixic acid resistance mutations in *E. coli* map to the structural genes for both gyrase subunits. Mutants have presumably lost the ability to bind nalidixic acid, and they can be isolated at high frequency (Miller 1972).

Finally, changes in bacterial cell permeability to an antibiotic can confer antibiotic resistance. Ampicillin resistance (Ap^r) in Gram-negative bacteria can arise from mutations in outer membrane proteins. Outer membrane porin proteins normally act as molecular sieves, allowing the diffusion of molecules into the cell according to charge and size. Ap^r mutants of this type have a reduced ability to transport the drug into the cell (Bush 1997; Dever & Dermody 1991).

The Luria-Delbrück fluctuation test is a classic quantitative measure of spontaneous mutation rate (Maloy et al. 1987). Multiple broth cultures of the bacterium under study are plated separately on selective medium. Individual mutant cells present in the broth cultures give rise to individual mutant colonies, while the nonmutant cells from each culture are killed. The fluctuation test derives its name from the observation that the separate cultures, each begun from a very small inoculum and each containing roughly the same number of cells, produce different numbers of mutant colonies. This fluctuation in mutant numbers indicates that mutation is a low probability event which occurs at random. Mutations occurring early in the growth of the broth culture will produce a higher mutant colony yield through the production of daughter cells before plating. This test assumes that the mutation under study is essentially neutral during growth in broth culture; that is, it does not create a growth advantage or disadvantage before plating on selective medium. This test also assumes that mutant reversion to the wild phenotype does not occur under normal testing conditions.

The mathematical derivation of the Luria-Delbrück equation calculating mutation rate is an adaptation of the Poisson probability distribution. The Poisson distribution quantifies the probability of success over an extremely large number of trials with a very small chance of success at each trial. A general formula for the Poisson probability function is:

$$e^{-m} (m)^x / x! = P(x)$$

where m = the average number of successes over a large number of trials; x = the number of successes, and $P(x)$ is the probability of observing x successes.

This probability function may be applied to the calculation of mutation rate as follows. Let us consider that a "success" in our system is the appearance of a mutant cell, and a "trial" is simply a cell division event. Let us also define N as the total number of cell division events. The quantity $N + 1$ therefore equals the total number of cells in a population derived from one parent cell. Since N is a very large number, it may be approximated by the total number of cells from one culture exposed to selective conditions during the experiment. Further, we define m as the mutation rate, the number of mutants produced during growth from one (nonmutant) cell divided by the number of cell division events. Consequently, units for m are mutants•division⁻¹. Under these circumstances, the quantity $N \cdot m$ represents the average number of mutants in a population derived from the ongoing division of one parent cell and its progeny. $N \cdot m$ is therefore functionally equivalent to the m of the Poisson distribution function, and we may make the following substitution:

$$e^{-Nm} (Nm)^x / x! = P(x)$$

Let us now consider the probability of getting no mutants at all ($x = 0$) after a large number of cell divisions. This greatly simplifies our equation since $(mN)^0$ and $0!$ are both equal to 1.

$$e^{-Nm} = P(0)$$

Taking the natural log of both sides gives:

$$-Nm \ln(e) = \ln P(0)$$

Since $\ln e = 1$,

$$-Nm = \ln P(0)$$

And finally,

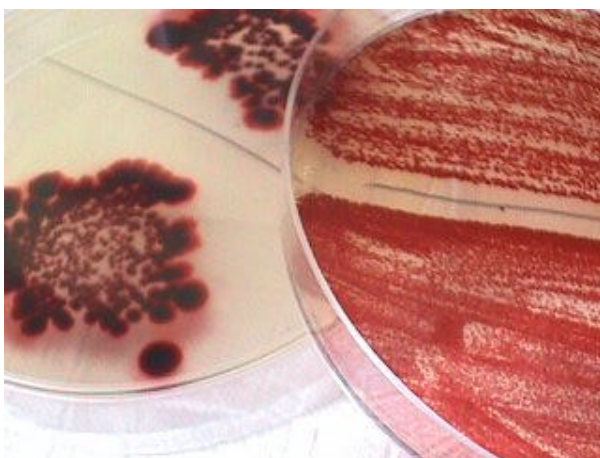
$$m = -(1/N) \ln P(0) \quad [\text{equation 1}]$$

Values for N and $P(0)$ must be experimentally determined. Again, N is approximated by the number of cells from each culture plated on selective medium. $P(0)$ is simply the fraction of cultures which give no mutant colonies on selective medium.

It should be noted that Luria and Delbrück (1943) derived two equations for measuring mutation rate. We have chosen the simpler equation, which relies only on values for N and $P(0)$ and not the number of mutants recovered from each culture. Equations which describe mutation rate as a function of the number of mutants recovered from each culture must assume that nonmutant and mutant bacteria multiply at the same

rate; this point must be verified experimentally. In fact, data to be presented elsewhere show that one class of Ap^r mutants multiplies significantly more slowly than nonmutant cells.

We have adapted the Luria/Delbrück fluctuation test to study the spontaneous mutation of the Gram-negative bacterium *Serratia marcescens* to ampicillin resistance at 25 micrograms antibiotic per ml (µg/ml). Our mutation system provides a fine laboratory introduction to the concepts of antibiotic resistance and spontaneous mutation, and it is easily adaptable to the advanced high school and undergraduate biology laboratories.



MATERIALS AND METHODS

Serratia marcescens NIMA (American Type Culture Collection # 29632) was used for all fluctuation experiments. Strain NIMA produces the red pigment prodigiosin, and it may be purchased inexpensively as “*S. marcescens* D1” from Carolina Biological Supply Company. *S. marcescens* was chosen for several reasons. *S. marcescens* is safe to handle; it is pathogenic for only immunocompromised individuals. This strain is naturally resistant to Sm (Williams & Gott 1964), and Sm was included in at 25 µg/ml in 1.5% agar plates as a precaution against unwanted bacterial contamination. Finally, our observation of a high rate of mutation to Ap^r makes *S. marcescens* an ideal choice for the student laboratory. In contrast, mutation of laboratory strains of *E. coli* to streptomycin resistance requires exposure of at least 10¹⁰ cells to selective conditions (Miller 1972). Such high cell numbers usually cannot be harvested without the extra step of centrifugation.

Serratia cells were grown in nutrient broth containing 0.5% maltose with shaking (aeration) at 30°C +/- 0.5°C using a Lab-Line model 3582 shaking water bath. Saturated cultures were diluted to produce an optical density at 620 nanometers (OD₆₂₀) of between 0.06 and 0.99 for calculation of cell density in a Beckman model DU 640B spectrophotometer. Other

work has shown that OD₆₂₀ readings vary directly with cell concentration within this optical density range (data not shown). The conversion factor 1.00 optical density unit = 5.80 x 10⁸ cells per ml was used for all bacterial cell quantitation. Dehydrated bacteriological media were purchased from VWR Scientific (Chicago, IL), and other chemical reagents were purchased from Sigma Chemical and Midwest Scientific (both of St. Louis, MO). Antibiotics were diluted from 1000X stocks in distilled water to a final concentration of 25 µg/ml. Antibiotics were added to cooled (ca. 55°C) media after sterilization by autoclaving at 121°C for 20 minutes on the slow exhaust cycle. Unwanted bacterial cultures were sterilized in the autoclave before disposal. The diluent phosphate buffered saline (PBS) was used at 1X after dilution from a 10X stock and sterilization. 10X PBS is 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, 2.4 g KH₂PO₄ in 1 liter of distilled water at pH 7.0 to 7.4.

S. marcescens was cultured in nutrient broth + 0.5% maltose. Maltose was included as the carbon source because nutrient broth + 0.5% glucose did not promote either growth to high cell density (OD₆₂₀ > 8.0) or strong prodigiosin expression (data not shown). Other rich media, such as LB (Miller 1972) can substitute for nutrient broth with maltose.

Each fluctuation test began with a pregrowth of *Serratia marcescens* in nutrient broth + 0.5% maltose. The single culture was shaken overnight at 30°C until strong prodigiosin expression was apparent by the culture’s dense growth and deep red color. This culture was subjected to serial tenfold dilution in sterile growth medium to give 1,000 to 10,000 cells per ml. 0.10 ml of the proper dilution, containing 100 to 1000 cells, was added to each of ten 125 ml Erlenmeyer flasks containing 10 ml of sterile medium. These cells constituted the parent populations for each culture. These ten flasks were shaken at 30°C until strong prodigiosin production was observed after approximately 24 hours, and they were harvested for plating as described below.

Two controls were included for the parent populations in each flask. Cells numbering ten times the parent inocula were plated on both Sm and Sm + Ap containing medium. These were the “parent population nonselective” and “parent population selective” controls, respectively. The nonselective control ensured the viability of the parent inocula, and the selective control assayed for the presence of mutants in these parent inocula.

After growth to high prodigiosin expression from less than 1000 cells, a sample from each culture was diluted up to 1:1000 into cold, sterile PBS on ice. This procedure was used to halt cell division before plating. A second sample of each culture was diluted 1:20 into sterile medium for spectrophotometric quantitation of cell number. The optical density measurement was used to calculate the volume of PBS-diluted culture

that would give the desired number of cells for plating. This volume, usually between 0.100 and 0.300 ml, was spread to dryness on a nutrient agar + 0.5% maltose + Sm plate. This plate served as a viability control, while a second plate additionally containing Ap served as the experimental selective plate. These volumes were plated using a 40-200-microliter (μ l) micropipettor with 1- μ l calibrations. Plates were incubated for approximately 24 hours at 30°C, and they were scored for type of growth and number of colonies.

For best results, *S. marcescens* should be grown with shaking (aeration) to high density in nutrient broth + 0.5% maltose or other rich medium. Deep red pigmentation is a good visual indicator of high cell density near stationary phase. Strict temperature control, however, is not needed because *S. marcescens* grows well and produces pigment at temperatures up to approximately 30°C. Dilution of dense, pigmented cultures before selective plating is necessary to increase the chance of obtaining no mutants for mutation rate calculations. The use of low temperatures to stop cell division before plating is not necessary under routine classroom conditions.

Teaching labs without access to a spectrophotometer for cell quantitation may still perform mutation rate measurements by estimating the

stationary phase cell concentration at 5.8×10^9 cells per ml. Begin the experiment by washing some growth from a fresh *S. marcescens* slant and inoculating each of ten cultures with 1 drop of cell suspension. Grow the cultures with shaking in nutrient broth + 0.5% maltose until strong prodigiosin expression is apparent. Dilute each culture 1000 fold into sterile medium or other diluent; the final cell concentration will be about 5.8×10^6 cells per ml. Spread 50 μ l (ca. 2.9×10^5 cells) onto nonselective (Sm) and selective (Sm and Ap) plates as usual.

RESULTS AND DISCUSSION

Table 1 shows the results of four independent fluctuation experiments. Data from experiments 2, 3 and 4 are useful for mutation rate calculations. The average spontaneous mutation rate of *S. marcescens* NIMA to resistance to 25 μ g/ml ampicillin was found to be 2.3×10^{-6} mutations per cell division. Viewed in a different way, this is twenty-three mutants produced during bacterial growth from a single cell to a population of ten million cells. Although all of our fluctuation experiments were done with ten cultures, the number of cultures which can be used for a given experiment is limited only by logistical constraints.

TABLE 1: FLUCTUATION TESTS FOR MUTATION OF *Serratia marcescens* TO 25 μ g/ml AMPICILLIN RESISTANCE. Numbers in the rows labeled "culture" represent colony counts on selective Sm + Ap plates. "TNTC" is used for resolvable colonies too numerous to count. "Lawn" indicates nearly uniform growth across the entire spread surface of the plate. A dash indicates no growth. Viability controls on nonselective (Sm) medium for all cultures in all experiments were positive for lawn or dense colony growth (data not shown). The value marked with an asterisk was calculated with an estimated minimal $P(0)$ value of 0.1.

Experiment #	1	2	3	4
# cells plated, N	5.0×10^7	1.0×10^6	5.0×10^5	3.0×10^5
Parent population nonselective control	Lawn	Lawn	Lawn	Lawn
Parent population selective control	-----	-----	-----	-----
Culture 1	313	7	3	9
Culture 2	332	7	1	1
Culture 3	331	2	1	--
Culture 4	TNTC	12	1	--
Culture 5	TNTC	--	1	1
Culture 6	245	4	1	1
Culture 7	255	5	1	--
Culture 8	TNTC	4	--	134
Culture 9	TNTC	14	--	--
Culture 10	TNTC	1	--	--
$P(0)$	< 0.1	0.1	0.3	0.5
mutation rate, m	$> 4.6 \times 10^{-8}$ *	2.3×10^{-6}	2.4×10^{-6}	2.3×10^{-6}

Table 1 also shows that the number of Ap^r colonies per culture generally decreases as the number of cells plated decreases. This result should be kept in

mind when designing classroom exercises. If the aim of the exercise is to show a fluctuation in the number of mutant colonies recovered, it is better to plate higher

numbers of cells (e.g., 1.0×10^7). Alternatively, if the aim is to calculate mutation rate, lower cell numbers should be plated (e.g., 1.0×10^5 to 1.0×10^6) so that some selective plates will show no growth. When 2.3×10^6 is used for m in equation 1, the useful range of values for N cells plated from ten cultures is 4.6×10^4 to 1.0×10^6 . Said another way, the number of cells plated from each of ten cultures should fall in this range in order to produce at least one plate with no mutants for the purposes of mutation rate calculation.

We have found that scoring the plates for mutant colonies is best done after 24 hours of incubation at 30°C. Cells which were mutant at the time of plating give rise to colonies of up to ca. 1 mm in diameter after 24 hours of incubation. Generally, mutant colony pigmentation is not well developed after 24 hours. At later times Ap^r colony pigmentation was variable, but most often the larger colonies were either red or white while the smaller ones were white.

Some selection plates which showed no growth up to 24 hours showed small colonies at later times. Because such colonies were probably derived from cells that were not Ap^r at the time of plating, they were not considered for the calculation of mutation rate. One recently studied selection system allows the development of microcolonies of initially nonmutant cells on selective plates (Andersson et al. 1998). The apparent mechanism giving rise to such cells is amplification of nonmutant genes which produces a low but sufficient level of mutant protein through

errors in translation. At some point during microcolony development mutation creates a cell which is capable of rapid growth under selective conditions; this leads to colony development. Perhaps a similar phenomenon is operative here.

We have adapted the Luria-Delbrück fluctuation test to measure the mutation frequency of a normally nonpathogenic organism, *Serratia marcescens*, to ampicillin resistance. This laboratory exercise is quantitative in nature, and it clearly illustrates the phenomenon of spontaneous mutation and its random occurrence. Theoretically, mutation rate measurements may be performed with any number of cultures; this exercise is readily adaptable for ten or more students. We use this system routinely in undergraduate biology laboratory classes, and it is our hope that this exercise can be adapted to the high-school biology classroom as well.

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