NATIONAL CENTER FOR CASE STUDY TEACHING IN SCIENCE

Which Came First, the Mutation or the Antibiotic? Understanding Antibiotic Resistance Through Fluctuation Analysis

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Part I – Sick in Samara

Phil, an undergraduate majoring in biology, was relaxing on the Quad and catching up with Facebook and email. He was hoping for a message from Dimitri, his Russian cousin, but once again nothing. How strange! Dimitri was normally very good about staying in touch. Something had to be wrong. As Phil lounged on the grass, he began to mull over some of the specifics that Dimitri had shared with him over the past few months. As a newly hired prison guard in the Samara region of Russia, Dimitri had recounted horrific details about the inmates' crowded and malodorous cells and recreational spaces. Many of the prisoners were sick with tuberculosis (TB), appearing feverish and coughing pus and blood; some were too weak to walk, and others paced with despairing looks in their eyes, sensing illness and death in their own near futures. In comparison, Dimitri felt very grateful for the three-room tenement apartment that he and his seven family members called home. He was often exhausted, telling Phil that his long overtime hours must be to blame. After several weeks of feeling more tired than usual, Dimitri had sent a short email to Phil, saying he must have some kind of chest cold, as he was now running a fever and had a cough but no sinus problems. As he grew more ill over the next few weeks, Dimitri had begun to recognize similarities between his symptoms and those of the prisoners he guarded. Upon Phil's urging, he had visited the local medical clinic. The results of the sputum cultures confirmed their worst fears: Dimitri had TB. He communicated this bad news to Phil over email, but reassured him that he was taking a six-month course of rifampicin and isoniazid, two first-line antibiotics. Dimitri strictly adhered to the schedule of treatment for three months, by which time he felt completely normal and well. He shared the good news with Phil, and told him that continuation of his antibiotic regimen was no longer justified. Before receiving Phil's cautionary reply, he sold the remaining doses of antibiotic on the black market in exchange for fuel to heat his family's cold apartment.

Now, two months later, Phil still hadn't heard from Dimitri and his anxiety would not be allayed until he called Dimitri's mother. Unfortunately, she confirmed his growing suspicions: Dimitri had antibiotic-resistant TB. One month after he had stopped taking the antibiotics, he had returned to the medical clinic, complaining of chest pain and blood in his sputum. His TB had returned and this time, the infection did not respond to the rifampin and isoniazid, even at higher doses. He was now a serious

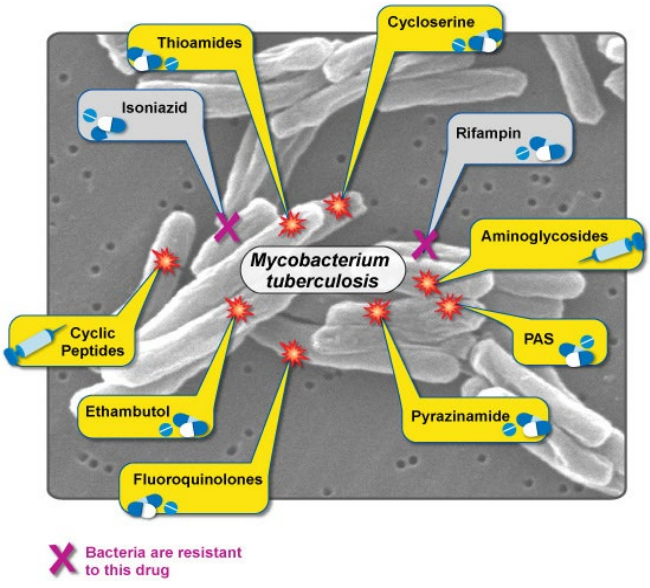

Figure 1. Antibiotic resistance in *Mycobacterium tuberculosis*. *Source:* http://www.niaid.nih.gov/topics/tuberculosis/Understanding/WhatIsTB/VisualTour/pages/mdr-tb.aspx.

public health risk and required treatment with second-line drugs such as fluoroquinolones and kanamycin. He was quarantined in a local medical facility in the hope that these drugs would work, despite rampant rumors that TB patients living in the Samara region were not responding to these second line drugs either.

Phil was distraught upon hearing this news. How did Dimitri acquire this antibiotic resistant form of TB? Did the first-line drugs they gave him worsen his TB somehow? Could the antibiotic resistance have been prevented? He texted his friend Stacy, and asked to meet with her at the café in the library. They were taking genetics this semester; maybe together they could determine what was happening with Dimitri.

Questions

Using the web resources listed below, answer the following questions.

1. What are the symptoms of TB?
2. How did so many inmates at the Samara prison, and Dimitri, likely contract TB?
3. Name two first line antibiotics used to treat TB.
4. If a patient's TB does not respond to first-line antibiotics, what type of TB does a patient have? What is the likely next step in treatments?
5. Explain how *Mycobacterium tuberculosis* can evolve resistance to first or second-line antibiotics. Describe the role of natural selection, and the role of physician and/or patient behaviors while taking antibiotics, in the evolution of antibiotic resistance.
6. Are there other antibiotics to which bacteria can become resistant? Name one.
7. What are the downstream repercussions of antibiotic resistance for the medical field as well as the general economy of countries?
8. Name a few specific practices that can be adopted to reduce the rate of evolution of bacterial antibiotic resistance.

Resources

Callaway, E. 2014. Russia's drug-resistant TB spreading more easily. *Nature News.* Retrieved October 2, 2014 from http://www.nature.com/news/russia-s-drug-resistant-tb-spreading-more-easily-1.14589.

Casali, N. et al. 2014. Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nature Genetics* 46, 279–286. Centers for Disease Control and Prevention. 2014a. Multidrug-Resistant Tuberculosis (MDR TB). Retrieved June 15, 2014 from http://www.cdc.gov/tb/publications/factsheets/drtb/mdrtb.htm.

Centers for Disease Control and Prevention. 2014b. Treatment of Drug-Susceptible Tuberculosis Disease in Persons Not Infected with HIV. Retrieved June 15, 2014 from http://www.cdc.gov/tb/publications/factsheets/treatment/treatmentHIVnegative.htm.

Centers for Disease Control and Prevention. 2014c. Treatment of Multidrug-Resistant Tuberculosis: Bedaquiline. Retrieved June 15, 2014 from http://www.cdc.gov/tb/publications/factsheets/treatment/bedaquiline.htm.

Levy, S.B. 2001. Antibacterial Household Products: Cause for Concern. *Emerging Infectious Diseases* 7, 512–515.

Understanding Evolution. University of California Museum of Paleontology. 2014a. Resisting our drugs. Retrieved June 15, 2014 from http://evolution.berkeley.edu/evolibrary/article/0_0_0/bergstrom_03.

Understanding Evolution. University of California Museum of Paleontology. 2014b. Turbo-charged evolution. Retrieved June 15, 2014 from http://evolution.berkeley.edu/evolibrary/article/side_0_0/turboevolution_01.

Understanding Evolution. University of California Museum of Paleontology. 2014c. Antibiotic resistance: delaying the inevitable. Retrieved June 15, 2014 from http://evolution.berkeley.edu/evolibrary/article/0_0_0/medicine_03.

Part II – Two Hypotheses

Stacy met Phil in the library café and immediately noted the anxiety written all over his face. After buying espressos, they sat down and Phil related Dimitri's story to her. Stacy observed that the fact that Dimitri's TB had responded to the first set of antibiotics suggested that he had acquired a *sensitive* form of TB, not a resistant form, from the inmates. In lecture, they had learned that antibiotic resistance is caused by mutations. So at some point after the initial infection, the *Mycoplasma tuberculosis* bacteria in Dimitri's lungs must have mutated and become resistant to certain antibiotics. Phil surmised that in Dimitri's case, his failure to take the full course of antibiotics had allowed a few bacteria to survive inside his lungs, despite the fact that he felt well. These bacteria no longer had any competition with other bacteria for food or other resources in the lungs and without the antibiotics the bacteria continued replicating. Sooner or later, a random mutation occurred that made them resistant to certain antibiotics. When the TB bacterial population had increased enough to cause symptoms, a fresh round of first line antibiotic therapy proved ineffective relative to the first time around.

Stacy realized that Phil was describing the "random mutation hypothesis," an example of natural selection driven by random mutation. However, Stacy had recently learned about the "acquired mutation hypothesis" and was not convinced by Phil's argument. She raised the alternative possibility that Dimitri's TB bacteria developed antibiotic resistance mutations to protect themselves after exposure to the antibiotics.

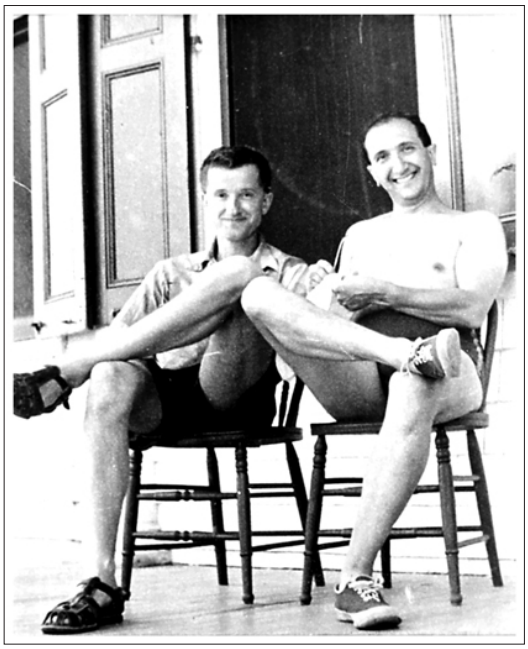

Figure 2. Max Delbrück and Salvador Luria, Cold Spring Harbor Laboratory, 1953. *Source:* U.S. National Library of Mediine, http://profiles.nlm.nih.gov/QL/B/B/J/F/_/.

Fortunately, the resolution to Phil and Stacy's argument would be achieved through that week's genetics lab experiment in which they would replicate the Nobel Prize-winning fluctuation test designed by Salvador Luria and Max Delbrück in the 1940s. Through this test, Luria and Delbrück had studied not antibiotic resistance, but bacteriophage resistance in bacteria. They presumed that mutations allowed bacteria to resist infection by this tiny bacterial virus but they did not know when the mutations occurred: before exposure to the bacteriophage (i.e., the "random mutation hypothesis"), or after exposure to the bacteriophage (the "acquired mutation" hypothesis). Another team of scientists, Esther and Joshua Lederberg had performed a very similar experiment around the same time, except that they studied antibiotic resistant bacteria. Stacy's professor suggested that they read the original papers written by Luria and Delbrück (Luria, S.E. and M. Delbruck. 1943. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28(6): 491–511) and the Lederbergs (Lederberg, J. and E.M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. *Journal of Bacteriology* 63(3): 399–406) to learn more about the experiments conducted by these scientists before conducting the experiment below.

Stacy and Phil's Replication of the Luria-Delbrück Fluctuation Test

A large test tube filled with liquid growth medium was inoculated with bacteria and grown for 16 hours to produce a large population of cells. The doubling time for the bacteria used was about 20 minutes. Twenty fresh culture tubes, each containing 1 mL of growth medium, were seeded with 100 bacteria from the large culture tube. A separate culture tube containing 10 ml of culture medium was also seeded with 100 bacteria from the large culture tube. All culture tubes were then incubated until each contained about 10^9 cells/ml. After shaking the culture tubes thoroughly, two experimental conditions A and B, which are described below, were created.

Condition A: From the separate culture tube described above, 10 subsamples of 1 mL were removed and spread onto solid agar media in 10 petri plates containing bacteriophage (a virus that will kill the bacteria). These plates are called *replicate plates*. All 10 plates were incubated for 16–24 hours and were scored for the number of colonies, all of which were mutant (i.e., resistant to the bacteriophage).

Condition B: The entire culture (i.e., 1 mL) from each of the 20 culture tubes was removed and spread on 20 individual agar plates containing bacteriophage. These plates are called *parallel plates*. All 20 plates were incubated for 16–24 hours and were scored for the number of colonies, all of which were resistant to the bacteriophage.

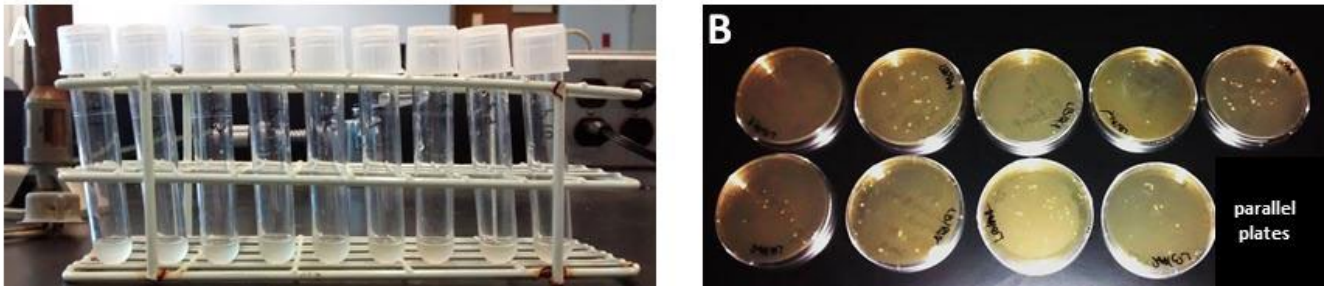

Figure 3. Examples of 1 mL bacterial cultures (A) and parallel agar plates (B). Each agar plate was spread with a single culture shown in (A). Note the different numbers of colonies on each plate, as indicated by the white dots.

The following data were obtained by Stacy and Phil's class (Table 1).

Table 1. Stacy and Phil's class data for the Luria-Delbrück fluctuation test based on *E. coli* resistance to bacteriophage.

Culture #	Condition A – # phage resistant mutant colonies on replicate plates	Condition B – # phage resistant mutant colonies on parallel plates
1	11	0
2	13	0
3	12	1
4	23	0
5	16	2
6	16	0
7	27	1
8	13	0
9	21	0
10	10	5
11		159
12		0
13		1
14		0
15		0
16		2
17		57
18		0
19		0
20		24

Questions and Instructions for Excel Simulation

Random Mutation Hypothesis

1. Under the *random mutation hypothesis* mutants will arise *randomly* in Stacy and Phil's culture tubes *prior* to being introduced to the bacteriophage on the plates.
   a. What would you then expect in terms of the consistency in the number of mutants in each culture tube at the end of the incubation period? *Why?*
   b. What will you expect to see in terms of the number of colonies observed on the *parallel* plates?
   c. What will you expect to see in terms of the number of colonies observed on the *replicate* plates?

Scientists often use simulations in an attempt to better understand the behavior of different phenomena, such as that being studied here. To assist you in visualizing what results might be expected under the random mutation hypothesis and the acquired mutation hypothesis, your instructor will provide you with an Excel spreadsheet. The spreadsheet has four tabs. For the following two steps, please focus on the first two tabs labeled "parallel plates (spontaneous)" and "replicate plates (spontaneous)."

Step 1: Open the sheet labeled "parallel plates (spontaneous)." Row 42 provides the number of colonies that might be found on each parallel plate under the spontaneous (random) mutation hypothesis. Rows 49–51 show the mean, variance and coefficient of variation for this experiment, respectively. Record these values in the first row of the table below.

	Parallel Plates (Spontaneous)	
Mean	*Variance*	*Coefficient of Variation*

Step 2: Open the sheet labeled "replicate plates (spontaneous)." Row 38 provides the number of colonies that might be found on each replicate plate under the spontaneous (random) mutation hypothesis. Rows 46–48 show the mean, variance and coefficient of variation. Record these values in the first row of the table below.

	Replicate Plates (Spontaneous)	
Mean	*Variance*	*Coefficient of Variation*

Now, repeat Steps 1 and 2 above at least one additional time. To do this:

PC Users: Click on the "FORMULAS" tab in the top toolbar and then click on the "Calculate Now" option, found to the far right in the top tool bar. This will simulate running the experiment a second time.

MAC Users: While holding down the FN key, depress the F9 key. This will simulate running the experiment a second time.

2. Upon completing this simulation, answer the following questions and then compare your responses to the answers you provided to Question 1 above. Do your answers differ from your initial responses, now that you have completed the simulation? What have you learned from doing this simulation?
   a. Under the random mutation hypothesis, what did you expect in terms of the consistency in the number of mutants in each culture tube at the end of the incubation period? *Why?*
   b. What did you see in terms of the number of colonies observed on the *parallel* plates?
   c. What did you see in terms of the number of colonies observed on the *replicate* plates?

3. While the above simulation is simplified it should be clear that under the *random mutation hypothesis* the variation in the number of mutant colonies appearing across the five *parallel* plates will be large/small (circle one), and the variation in the number of mutant colonies appearing across the five *replicate* plates will be large/small (circle one).

4. Under the random mutation hypothesis, we can conclude that the number of mutants in the culture tubes used to seed these plates is *primarily dictated* by: (Select one of the following.)
   a. The timing of when the first mutant occurred in that culture tube.
   b. The temperature of incubation.
   c. The number of culture tubes.
   d. The number of generations observed.

Acquired Mutation Hypothesis

5. Under the acquired mutation hypothesis mutants will arise at regular intervals only after being introduced to an agent, like the bacteriophage, that requires them to adapt.
   a. What would you expect in terms of the number of mutants in each culture tube at the end of the incubation period? *Why?*
   b. What will you expect to see in terms of the number of colonies observed on the *parallel* plates?
   c. What will you expect to see in terms of the number of colonies observed on the *replicate* plates?

Again, use the Excel spreadsheet to assist you in visualizing what results might be expected under the acquired mutation hypothesis.

Step 3: Open the sheet labeled "parallel plates (acquired)." Row 42 provides the number of colonies that might be found on each parallel plate under the acquired mutation hypothesis. Rows 49–51 show the mean, variance and coefficient of variation. Record these values in the first row of the table below.

	Parallel Plates (Acquired)	
Mean	*Variance*	*Coefficient of Variation*

Step 4: Open the sheet labeled "replicate plates (acquired)." Row 38 provides the number of colonies that might be found on each replicate plate under the acquired mutation hypothesis. Rows 46–48 show the mean, variance and coefficient of variation. Record these values in the first row of the table below.

	Replicate Plates (Acquired)	
Mean	*Variance*	*Coefficient of Variation*

Repeat Steps 3 and 4 above at least one additional time, using the instructions for PC/MAC users previously given.

6. Upon completing this simulation, answer the following questions and then compare your responses to those you provided in Question 5 above. Do your answers differ from your initial responses now that you have completed the simulation? What have you learned from doing the simulation?
   a. Under the acquired mutation hypothesis, what did you expect in terms of the number of mutants in each culture tube at the end of the incubation period? *Why*?
   b. What did you see in terms of the number of colonies observed on the *parallel* plates?
   c. What did you see in terms of the number of colonies observed on the *replicate* plates?

7. Using the simulation as a guide it should be clear that under the *acquired mutation hypothesis* the variation in the number of mutant colonies appearing across the five *parallel* plates will be large/small (circle one), and the variation in the number of mutant colonies appearing across the five *replicate* plates will be large/small (circle one).

The Actual Experment

8. Now let's examine the data that was collected from the actual experiment performed by Phil and Stacy's class. (See Table 1 above). Calculate the mean, variance and coefficient of variation for Condition A. Repeat for Condition B. Clearly, the coefficient of variation for Condition A (the replicate plates) is much larger/smaller (circle one) than that of condition B (the parallel plates).

Condition	*Mean*	*Variance*	*Coefficient of Variation*
A			
B			

9. It appears that the data collected supports (circle one):
   a. the random mutation hypothesis.
   b. the acquired mutation hypothesis.
10. The random mutation hypothesis then is consistent with (circle one):
    a. the variance between Condition A and Condition B will be approximately the same.
    b. the variance between Condition A and Condition B will be markedly different.
11. Hypothesis testing requires that one reject the null hypothesis in order to accept the alternative hypothesis. Given our discussion above one might be inclined to set up a hypothesis test to statistically compare the variance in Condition A versus the variance in Condition B. What might the null hypothesis and the alternative hypothesis be?

Part III – Mutation Rate

Stacy and Phil's class experiment revealed that the bacteriophage resistance mutations arose before bacteria were exposed to the bacteriophage, so the random mutation hypothesis had been supported. Applying their new-found knowledge to Dimitri's situation, Phil and Stacy realized that a small number of TB bacteria probably had become resistant around the time that he had stopped taking the antibiotics. Unfortunately, these resistant bacteria replicated undeterred until the TB symptoms recurred.

Something still bothered Phil, however. He understood that random mutation can happen and could lead to antibiotic resistance, but he questioned how often random mutations occur. From poor Dimitri's case, it seemed that lots of mutations must have occurred. Stacy reminded Phil that mutations are rare. Their professor had asked them to calculate the mutation rate at the end of the experiment, but Phil had missed class that day. She offered to show him the calculations.

Math Concepts

Under the assumption that bacteria divide approximately every 1/3 hour (20 minutes), the growth of bacteria follows the exponential model: $y = 8^t$, where y represents the population of bacteria at time t hours, and the base of 8 (or 2^3) reflects the number of cells present after 1 hour from 1 initial cell, with a doubling time of approximately 1/3 hour (see Figure 4).

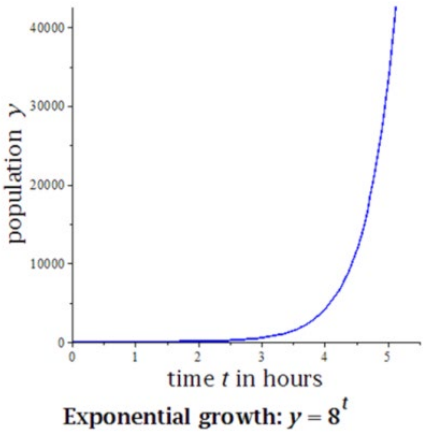

Figure 4. Exponential growth, $y = 8^t$.

As the occurrence of a mutant from a non-mutant cell can be considered a "rare" event, the Poisson Probability Distribution (see Luria and Delbrück, 1943) can be used to compute the probability of mutations occurring in a given volume of the culture. Therefore we describe here the calculation of mutation rate using the Poisson distribution.

The Poisson Probability Distribution is described mathematically as:

$$P(x, \mu) = \frac{e^{-\mu}\mu^x}{x!}$$

This discrete probability distribution applies to the occurrence of some event over a specified interval. The interval can be time, distance, area, volume or some similar unit. The Poisson distribution satisfies the following conditions:

1. The random variable x is the number of occurrences of the event in the interval of interest.
2. μ is the historical average of occurrences for the interval of interest.
3. The occurrences must be random.
4. The probability of the event occurring is the same for each interval.
5. The number of occurrences in one interval is independent of the number of occurrences in other disjoint intervals. (See Larson and Farber, 2014.)

The shape of the Poisson distribution is determined by the mean, μ, the average number of occurences of the event in the given interval (see Figure 5).

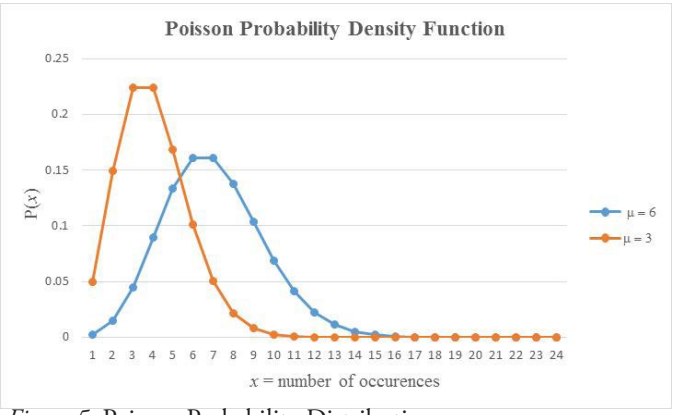

Figure 5. Poisson Probability Distribution.

Formula for the Calculation of Mutation Rate

The notations given below, including the mutation rate formula are derived from Luria and Delbrück, 1943. The Poisson Probability formula given below can be found in Larson and Farber, 2014. In the Luria-Delbrück experiment, the interval used is the given volume of culture.

N_0	The initial population of bacteria, i.e., population at time $t = 0$ in a given volume of culture. Note that $N_0 \leq 100$ in our experiment.
N_t	The final population of bacteria in a given volume of culture.
$N_t - N_0$	The number of cell division events from N_0 parent cells in a given volume of culture. (Since N_t is a very large number, $N_t - N_0 \approx N_t$, the total number of cells in a given volume.)
p	The mutation rate (the probability that a mutation occurs when a cell divides).
μ	The average or expected number of mutations in a given volume of culture derived from the ongoing division of N_0 parent cells and their progeny by time t.

The mutation rate p can be calculated as the ratio of the expected number of mutations produced to the number of cell divisions in a given volume of culture, i.e., $p = \frac{\mu}{N_t}$. This implies that $\mu = p \cdot N_t$.

Therefore, by the Poisson probability formula, the probability of observing x mutations in a given volume of culture is, approximately:

$$P(x, \mu) = \frac{e^{-\mu}\mu^x}{x!} = \frac{e^{-p \cdot Nt}(p \cdot N_t)^x}{x!}$$

Let $P(0) = P(0, \mu)$ denote the probability of observing no colonies on a plate. Since $(p \cdot N_t)^0 = 1$ and $0! = 1$, we have:

$$P(0) = e^{-p \cdot Nt}$$

Then $\ln P(0) = -p \cdot N_t$, implying that

$$p = -\frac{\ln P(0)}{N_t}$$

The values of $P(0)$ and N_t will be experimentally determined as follows: To calculate $P(0)$, the probability of observing no mutations in a series of parallel cultures, divide the number of parallel cultures with 0 mutations by the total number of parallel cultures. To calculate N_t, the total number of bacteria in a plate, multiply the total number of bacteria/ml in a given volume of culture by the volume spread on the plate. *Note that to calculate mutation rate in this manner it is necessary to obtain some plates with no colonies.*

Questions

The questions below lead to the calculation of the mutation rate for the experiment. Please refer to the math concepts above to answer these questions.

1. Identify the criteria required for a Poisson distribution and determine why the Poisson distribution serves as an adequate probability model to calculate the probability of x mutations in a culture.
2. State the Poisson probability formula/equation for calculating the probability of x mutations in a culture.
3. Using the formula in Question 2, find the formula for $P(0)$, the probability of getting no mutations at all in a culture.
4. State the formula for μ, the mean or expected number of mutations in a culture, in terms of the mutation rate p and the final population of bacteria N_t.
5. Using the formulas in Questions 3 and 4, derive the formula for the mutation rate p in terms of N_t and $P(0)$.

6. In the experiment, what is N_t, the final population of bacteria in the culture spread on agar plates?
7. Use the data from the experiment to calculate $P(0)$, the probability of getting no mutations among parallel cultures.
8. Calculate the mutation rate for the experiment using the information from Questions 5–7 and explain what it means.

Conclusion

Having worked through the mutation rate calculations, Phil now appreciated how the statistics and functions that he had learned in his math classes were relevant to his life and could be applied to real biological problems. Phil couldn't help asking Stacy to clarify one more point: he didn't understand how rare mutations could have made Dimitri so sick.

Stacy reminded him that bacteria divide about every 20 minutes (one generation), and at each division there is a very low but finite chance of mutating. Bacteria will divide exponentially for many generations before an infection is detectable, so many resistant bacteria can be present, especially if the resistance mutation arose early in the population.

Phil sighed. His cousin Dimitri had suffered from a combination of bad luck and poor judgment. The next moment, however, he brightened visibly as he glanced at his emails. Dimitri had finally written, and he was reporting good news; he was responding to the rigorous course of second line antibiotics. The two let out sighs of relief. They were now hopeful that Dimitri was going to overcome this terrible infection. Their eyes now opened about the dangers of antibiotic resistance, Stacy and Phil thought that it was high time that they got the message out to the student body. "Take your full course of antibiotics or you too could succumb to mutants!"

Additional Resources

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World Health Organization. 2014. Antimicrobial resistance: global report on surveillance 2014. Retrieved June 15, 2014 from http://www.who.int/drugresistance/documents/surveillancereport/en/.