Natural selection and evolution of antibiotic resistance in bacteria

(adapted from: Pryce L. Haddix, Eric T. Paulsen and Terry F. Werner, Bioscene, v26, p1-21, Feb 2000)

BACKGROUND

"Nothing in biology makes sense except in the light of evolution" - Theodosius Dobzhansky

In popular literature evolution is often discussed in the context of events that happened long ago and when many people think about evolution they focus on fossils and what they tell us about the past. While it is true that evolutionary theory seeks to explain relationships between species and to understand their origins few realize that evolutionary theory is also used to predict what will happen in the future. Evolution is the one theme which underlies and unites all off biology, and while we rarely take note, all of the living things that surround us continue to evolve. It is usually easiest to observe evolution at work in species that have very short generation times or that have very many progeny. Just ask any farmer. Insecticides and herbicides were first introduced in the 1940s and 1950s and it wasn't long before this selection pressure began to effect the number of resistant varieties of plants and insects in the world. Since insects and weeds cost farmers money the farmers take a great deal of interest in understanding how to manage the evolution of pest resistance. Today, the way in which farmers use herbicides and pesticides has in many ways been shaped by our understanding of evolutionary theory in order to minimize the ability of pests to evolve resistance.



Evolutionary theory also plays a major role in medicine and there is no better example of this then the evolution of antibiotic resistance in bacteria. Prior to the introduction of antibiotics in the 1940s antibiotic infections were rare but there frequency increased rapidly as antibiotic use increased. In fact, most of the antibiotics which were first used in the 1940s and 1950s are no longer used clinically because resistance to these antibiotics is now so commonplace. New antibiotics were developed over time and with the introduction of each new medicine resistant bacteria quickly appeared. Today, we have changed the way that antibiotics are used and prescribed to try to slow the relentless pace of bacterial evolution, but a solution to this problem has yet to be found. Microbiologists continue to study how bacteria evolve so that we may predict how microbes will respond to medical treatments and we can better manage the evolution of infectious disease.

Antibiotics have many different modes of action and as a result bacteria can evolve resistance to antibiotics in several different ways. In general, bacterial resistance to antibiotics takes one of three forms: 1) A change in the permeability of the cell membrane which either prevents the entry of the antibiotic into the cell or causes the antibiotic to be pumped out of the cell, 2) Acquiring the ability to degrade and/or inactivate the antibiotic, or 3) Acquiring a mutation that modifies the target of an antibiotic so that the target is no longer sensitive. There are two ways that bacteria can acquire resistance to antibiotics. The resistance genes can arise spontaneously in a population of cells as a result of mutation

or in certain cases they can be acquired from a different microorganism through a process called Horizontal Gene Transfer. In Horizontal Gene Transfer a gene or genes can be exchanged between unrelated microbial species.

In our exercise today a single colony was used as the starting material to grow a culture of bacteria. Since one colony generally forms from a single cell all of the cells in our culture have the same mother cell and if not for random mutation would be genetically identical. Thus, genetic variability within the population of cells in our culture can only result through the accumulation of random mutations. We will observe that as a result of random mutation a small number of cells within our bacterial population (about 1 in a million) will become resistant to the antibiotic ampicillin. If no selection pressure is applied resistant cells will always remain rare in the population. However, the situation changes if we transfer the culture to growth media containing ampicillin. The ampicillin provides a strong selective pressure which allows resistant cells to grow but which kills off cells that are not resistant. This selective pressure causes a dramatic increase in the frequency of resistant cells within the population of cells in the culture. This is a demonstration of the underlying principles of natural selection, natural variation exists in the population as a result of the random accumulation of mutations and selection causes the most fit individuals to increase in frequency.

<u>MATERIALS</u> (listed amounts are for 14 groups of 2)

- 1 Serratia marcesens D1 (ATCC# 29632) (available from Carolina Biological)
- 1.5 L Sterile 1X PBS (12 g NaCl, 0.3 g KCl, 2.16 g Na2HPO4, 0.36 g KH2PO4 in 1.5 L water)
- 160 Glass or plastic test tubes (about 20-30ml volume, with closure that can breathe (not screw caps))
- 80 Sterile Petri Plates
- ~175 Sterile 1 ml transfer pipettes or tips (1ml and 10ul)
- ~175 Sterile droppers or 10 ul tips (10 ul is about one small drop)
- 14 Spreaders (can be made by bending a glass rod or pipette over a Bunsen burner)
- 25 Tubes of 10 ml nutrient broth + 0.5% maltose
- (nutrient broth can be purchased as powder and rehydratred, maltose is purchased separately) $\Delta \alpha \alpha r$
- 24g Agar
- 2 ml 25 mg/ml solution of ampicillin
- 2 ml 25 mg/ml solution of streptomycin (optional)
- ~0.5 L 70% EtOH
- 14 Bunsen burners

PRIOR EXPERIMENTAL SETUP

Sterilze glassware. Glassware can be sterilized in a steam autoclave for 20 minutes at 15 psi or by baking empty glassware in an oven at 350° F for 2 hrs. Tops of tubes and bottles should be sealed with aluminum foil to prevent contamination. Glass pipettes should be in glass or metal containers covered with aluminum foil or bundled in foil packets.

Prepare liquid media. Add the appropriate amount of nutrient broth powder (typically 8g per L) to 250 ml water then add 1.25 g maltose (5 g per L). Sterile in a steam autoclave for 20 minutes at 15 psi or by boiling for 30 minutes. Note boiling will not result in complete sterility but should be sufficient for most teaching purposes; to minimize potential contamination place media in a pre-sterilized glass container, seal with foil, and then place in water and bring to a boil. Begin timing after the water has started to boil. Dispense 10 ml by sterile pipette into 25 sterile glass tubes with closures. When cool add 10 ul (about one small drop from a dropper) of 25 mg/ml ampicillin to 1 tube for each group. *Serratia marcesens* D1 is naturally resistant to streptomycin

and so to reduce the chance of contamination you can optionally also add 10 ul of 25 mg/ml streptomycin to all tubes.

Prepare PBS dilution blanks. Prepare 1.5 L of 1XPBS. Dispense 9ml into 140 test tubes and sterilize by autoclaving. If boiling, then boil first and when sterile dispense aseptically into pre-sterilized test tubes.

Prepare agar plates. Prepare 500 ml and 1,250 ml of nutrient broth + 0.5% maltose and add 15 g/L of agar to each. Sterilize as described above. Allow to cool until you can just hold your hand against the bottle for about 3-5 seconds (~60° C). If streptomycin is desired add 1 ml per L of 25 mg/ml stock at this time and mix well. Pour ~20 plates from the 500ml portion and label -Ap. Add 1 ml per L of the 25 mg/ml ampicillin stock to the 1,250 ml portion and mix well. Pour ~60 plates from the 1,250 ml portion and label +Ap. (**Note**: For best results the agar surface needs to be "dry". Fresh plates or plates that are fresh out of a damp refrigerator can sometimes have a surface film of water that causes bacterial colonies to smear. To be sure that plates are dry leave them out at room temperature for one or a few days or place them in an incubator for a few hours or overnight before they are needed.)

Prepare the culture. Streak *S. marcesens* out onto a nutrient agar -Ap plate and incubate overnight at 30° C (or about 2 days at room temperature). Pick one colony into 10 ml fresh nutrient broth -Ap, grow 30° C shaking 24 hrs (this should yield approximately 5×10^9 cells) or >24 hrs at room temperature. Use 0.5 ml of this starter culture to inoculate one 10 ml nutrient broth –Ap parent cultures for every 2-4 lab groups.

EXPERIMENTAL METHOD

<u>Day 1 (15 min)</u>

Counting the number of resistant bacteria in the parent culture.

1) To do this exercise you will need to pair up with another group. One group will be called **Group A** and the other group will be called **Group B**.

2) All groups should Label 4 sterile tubes of 9 ml PBS: tube 1 through tube 4. Transfer 1 ml of the parent culture to tube 1 mix well, then transfer 1 ml of tube 1 to tube 2. Continue sequentially in this manner until you have carried the dilution through all 4 tubes.

2) All groups should use a marker to divide 1 nutrient agar +Ap plate into 5 equal wedges and label the wedges 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} .

3) All groups should place three evenly spaced spots of 10 ul from the parent culture into the wedge labeled 10^{-2} . Next, place three evenly spaced spots of 10 ul from tube 1 into the wedge labeled 10^{-3} . Next place three evenly spaced spots of 10 ul from tube 2 into the wedge labeled 10^{-4} . Continue in this fashion until all of the wedges have been used. Be careful not to move the plate until the spots have dried. It may take several hours for the spots to dry completely and dry to prevent dripping or smearing the plates should be handled carefully and not inverted until the spots are dry. Label the plate 'parent +Ap'.

4) **Group** A should inoculate a 10 ml tube of nutrient broth + 0.5% maltose with 100 ul of the parent culture. Label the tube 'Group A.

5) **Group B** should inoculate a 10 ml tube of nutrient broth + 0.5% maltose + 25 ug/ml Ampicillin with 100 ul of the same parent culture. Label the tube 'Group B'.

6) Incubate the plates and tubes 24 hrs at 30° C or 2 days at room temperature. Tubes should be aerated by shaking vigorously.

Day 2 (30-40 min)

Counting the number of resistant bacteria after natural selection.

Group 'A'

1) Label 8 tubes of sterile PBS tube 1 through tube 8. Transfer 1 ml of the +Ap culture to tube 1 mix well, then transfer 1 ml of tube 1 to tube 2. Continue sequentially in this manner until you have carried the dilution through all 8 tubes.

2) Use a marker to divide 1 nutrient agar plate without ampicillin into 5 equal wedges and label the wedges 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} .

3) Place three evenly spaced spots of 10 ul from tube 4 into the wedge labeled 10^{-6} . Next, place three evenly spaced spots of 10 ul from the tube 5 into the wedge labeled 10^{-7} . Next place three evenly spaced spots of 10 ul from the tube 6 into the wedge labeled 10^{-8} . Continue in this fashion

until all of the wedges have been used. Be careful not to move the plate until the spots have dried. It may take several hours for the spots to dry completely and dry to prevent dripping or smearing the plates should be handled carefully and not inverted until the spots are dry. Label the plate 'Group A -Ap'

4) Use a marker to divide 1 nutrient agar plate with 25 ug/ml ampicillin into 5 equal wedges and label the wedges 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} .

5) Place three evenly spaced spots of 10 ul from the culture into the wedge labeled 10^{-2} . Next, place three evenly spaced spots of 10 ul from the tube 1 into the wedge labeled 10^{-3} . Next place three evenly spaced spots of 10 ul from the tube 2 into the wedge labeled 10^{-4} . Continue in this fashion until all of the wedges have been used. Be careful not to move the plate until the spots have dried. It may take several hours for the spots to dry completely and dry to prevent dripping or smearing the plates should be handled carefully and not inverted until the spots are dry. Label the plate 'Group A +Ap'

6) Incubate the plates overnight at 30° C or 2 days at room temperature.

Group 'B'

1) Label 8 tubes of sterile PBS tube 1 through tube 8. Transfer 1 ml of the +Ap culture to tube 1 mix well, then transfer 1 ml of tube 1 to tube 2. Continue sequentially in this manner until you have carried the dilution through all 8 tubes.

2) Use a marker to divide 1 nutrient agar plate without ampicillin into 5 equal wedges and label the wedges 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} .

3) Place three evenly spaced spots of 10 ul from tube 4 into the wedge labeled 10^{-6} . Next, place three evenly spaced spots of 10 ul from the tube 5 into the wedge labeled 10^{-7} . Next place three evenly spaced spots of 10 ul from the tube 6 into the wedge labeled 10^{-8} . Continue in this fashion until all of the wedges have been used. Be careful not to move the plate until the spots have dried. It may take several hours for the spots to dry completely and dry to prevent dripping or smearing the plates should be handled carefully and not inverted until the spots are dry. Label the plate 'Group B -Ap'.

4) Use a marker to divide 1 nutrient agar plate with 25 ug/ml ampicillin into 5 equal wedges and label the wedges 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} .

5) Place three evenly spaced spots of 10 ul from tube 4 into the wedge labeled 10^{-6} . Next, place three evenly spaced spots of 10 ul from the tube 5 into the wedge labeled 10^{-7} . Next place three evenly spaced spots of 10 ul from the tube 6 into the wedge labeled 10^{-8} . Continue in this fashion until all of the wedges have been used. Be careful not to move the plate until the spots have dried. It may take several hours for the spots to dry completely and dry to prevent dripping or smearing the plates should be handled carefully and not inverted until the spots are dry. Label the plate 'Group B +Ap'

6) Incubate the plates overnight at 30° C or 2 days at room temperature.

Day 3 (10-15 min)

1) count colonies from the plates that you made on Days 1 and 2 and enter results below:

Culture	Plate	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10^{-10}
Parent	+Ap						n.a.	n.a.	n.a.	n.a.
Group A	+Ap						n.a.	n.a.	n.a.	n.a.
Group A	-Ap	n.a.	n.a.	n.a.	n.a.					
Group B	+Ap						n.a.	n.a.	n.a.	n.a.
Group B	-Ap	n.a.	n.a.	n.a.	n.a.					

QUESTIONS

What is the frequency of resistant cells in the parent culture?

What is the frequency of resistant cells in the +Ap culture? Why are there more resistant cells in the +Ap culture than in the -AP culture?

Was the total number of cells present in the +Ap and –Ap cultures the same? Why or why not?

The parent culture was started from a single cell do you think this ancestor was resistant to Ap?

How do you think the cells became resistant?

In what ways does this experiment parallel the evolution of plants and animals and in what ways is it different?

FURTHER EXPLORATIONS

-You can observe variation in the size of colonies from the parent culture or the –Ap culture when grown on the +Ap plates. What causes this variation? Do you also observe the same amount of variation in colony size when the +Ap culture is place on the +Ap plates? You can investigate further by picking different sized colonies from the plates and streaking them out to see if the size differences persist. If you streak out the different sized colonies and the difference in colony size persists then try inoculating +Ap liquid media to see how long it takes for the different cultures to become turbid (this will give you an idea if there is a difference in growth rate). Also test the isolates for growth in the presence of different amounts of antibiotic. Do you think there is only one type of mutation that can result in resistance or are there many different ways that an organism may become resistant?

-You can observe that the +Ap culture is not as turbid as the –Ap culture after 24 hrs of growth. Why do you think that is? Are you adding the same number of viable cells to both cultures the at the start of the experiment or does one have more viable cells than the other? Do you think that even though the antibiotic doesn't kill the resistant cells that it still may slow their growth? Or might the mutation that induces resistance have a cost to the cell that limits its growth rate (for example the mutation that conferred resistance may have occurred in a gene that helps the cell to grow fast so that the same mutation that confers resistance also slows growth). What experiments might you perform to find out what is going on?

EXPECTED RESULTS

The rate at which S. marcesens develops resistance to ampicillin is approximately 1 resistant cell per 1 x 10^6 cells in the population. A culture S. marcesens will have ~5 x 10^9 cells per ml in stationary phase (when growth is complete). Thus, you would expect about 5000 resistant cells to be present per ml of culture. Since the parent culture was started from a single colony which itself was started from a single cell all of the cells in the culture share the same ancestor (which was sensitive to Ampicillin) and any variation that has arisen must be the result of mutation. By dividing the number of colonies by the dilution you can find the number of cells (ex: 5 colonies / $10^{-9} = 5 \times 10^{9}$ cells). In the table below, Row A tells you how many cells were resistant (per ml) in the parent culture, row B the number of resistant cells present after a selective pressure was applied, row C the total number of cells present after selection (resistant + sensitive), row D the number of resistant cells present when there is no selection, and row E the total number of cells present in the absence of selection. You can determine that the mutation rate is $\sim 1 \times 10^{-6}$ by dividing the number of cells per ml from row D/E. In addition since ampicillin kills bacteria the number of cells per ml in B/C should ~ 1. Thus the number of resistant bacteria in the population has increased by a million fold as a result of the selective pressure that we applied. In the absence of the selective pressure the population continues to resemble the parent culture (row D compared to row A).

	Culture	Plate	10 ⁻²	10^{-3}	10 ⁻⁴	10 ⁻⁵	10-6	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰
Α	Parent	+Ap	TNTC	50	5	0	0	n.a.	n.a.	n.a.	n.a.
В	Grp A	+Ap	TNTC	50	5	0	0	n.a.	n.a.	n.a.	n.a.
С	Grp A	-Ap	n.a.	n.a.	n.a.	n.a.	TNTC	TNTC	50,40,60	5,6,4	0
D	Grp B	+Ap	n.a.	n.a.	n.a.	n.a.	TNTC	TNTC	50,40,60	5,6,4	0
Е	Grp B	-Ap	n.a.	n.a.	n.a.	n.a.	TNTC	TNTC	50,40,60	5,6,4	0

In addition, you will make two additional observations. First, when you plate the parent culture (or the –Ap culture) on +Ap plates you will see a lot of variation in the size of the colonies that form on the +Ap plate relative to the sizes of the colonies that you see on –Ap plates. The variation in size of the colonies results from the fact that different mutations can result in different fitness in the presence of the antibiotic. More 'fit' cells will form bigger colonies. Also, some cells may mutate while they are sitting on the plate in the incubator (as opposed to when they were in culture) and may start to form colonies after the mutation has occurred. You should only count the larger colonies (~1mm or larger in diameter after 24hrs growth at 30C) to get the number of resistant cells per ml of culture that was plated. Second, the +Ap culture will grow more slowly than the –Ap culture.

REFERENCES

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