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Introduction

This exercise was developed to complement lectures on evolution. Conceptually, it aims to challenge student to think through the relationship between mutations as random sources of variation, and adaptation which results from selection working on variants and which is decidedly non-random. In particular, the exercise seeks to clear up a misconception which students seem to have about how new variation arises in a population — adaptive variants do not arise because of the particular environment, rather they fortuitously prove to be adaptive after they arise. The exercise is based on original research by Lederberg and Lederberg (1952) which used replica-plating to test competing hypothesis about the origin of adaptive mutants in *E. coli*.

The exercise as presented here is suitable for an introductory class, but it could be used at higher levels if the microbiological procedures, such as sterile techniques, are made more rigorous. The exercise has three parts which take about 2 weeks to complete; the total laboratory contact time is about 3 hours (1 hour per part). During the first lab students learn to pour an agar plate and inoculate it with a yeast suspension. Each students takes their yeast culture home. After 3 days of growth (at room temperature), the culture is refrigerated to stop further growth until the next lab. At the second lab, students make replica plates of the primary culture onto agar plates containing copper. The replica plates are cultured at home for about 5 days until mutant colonies are well formed; the plates are refrigerated until the third lab. In the final lab, the spatial patterns on both replica plates are compared as a test of the initial hypotheses. At the University of Toronto, where labs are held every second week, this exercise continues over 6 weeks.

Materials

Each student will need the following:

Agar (20 ml)

Agar with copper added (40 ml)

Baker's yeast (*Saccharomyces cerevisiae*), packaged, dry (0.1 g)

Petri plate, 15 × 100

Brass spreader

Ethanol, 70%, in a washbottle (to clean equipment)
 Pasteur pipet
 Shell vials (for serial dilution) (2)
 Flask, erlenmeyer (for yeast suspension)
 Marking pen
 Masking tape
 Pipet, 10-ml “to deliver”
 Propipetter
 Replica plater
 Velvet square, 15 × 15 cm, sterile
 Physiological saline (0.85% NaCl), autoclaved (50 ml)
 To make 1 litre of agar add: DIFCO neopeptone (10 g), bacto-dextrose (40 g), bacto-agar (20 g), and water (1 liter).
 For 1 liter of 3 mM copper agar, add: copper sulphate (0.48 g).

Student Outline

Objectives

1. Conduct an experiment to test when copper-tolerant mutants arise in a yeast population.
2. Learn how to culture and handle yeast.
3. Identify mutant cells using the replica-plating technique.

Preparation

Before you come to the lab, read the sections on fungi (pages 509–511), mutation (pages 261–265), and replica plating (page 273) in the course textbook, Purves et al. (1992).

Introduction

When do adaptive mutants arise in a population? Mutation provides new variation in a population. New variation is essential to a population's long-term survival, since it can provide new adaptations if the environment changes. However, mutations at any particular gene occur very infrequently. How then can evolution adapt the population quickly enough in a new environment to ensure the continued survival of the population?

One possibility is that mutations happen spontaneously and so there is always potentially useful variation present in a population. If the environment changes some of the pre-existing mutations might be favoured by selection and increase to dominate in the population. This is the process of local adaptation.

Another possibility is that useful mutations are induced to occur in some individuals only after the environment has changed. In this exercise, you will conduct an experiment to test whether copper-tolerant mutant yeast cells arise spontaneously and pre-exist in a normal population, or whether they are induced by exposure to copper.

Replica Plating

Replica plating is a standard microbiological technique for isolating mutant cells, and it can help us to test when adaptive mutants arise in the yeast population. In replica-plating, a flat, velvet surface is used like a stamp to pick up cells from colonies on an original plate (usually colonies growing on normal media). The cells can then be transferred to another plate (usually containing chemicals which only the mutant cells will survive on) in the *exact same spatial arrangement* as they were on the original plate.

Culturing Yeast

Baker's yeast, *Saccharomyces cerevisiae*, is a small oval fungus which reproduces quickly (cells divide about every 8 hours under optimal conditions) and so is an ideal organism for mutation experiments. Yeast normally live on the surface of fruit and leaves. Humans use yeast in baking and brewing. In the lab, you will grow yeast on a sterile, solid agar medium. Agar is a polysaccharide derived from red seaweeds (see page 497 in Purves, 1992). Agar is a good gelling agent because it is not degraded by microbes and it is stable over a wide range of temperatures.

To prepare agar media, a liquid culture medium is mixed with 2% agar and then brought to a boil to dissolve the agar. The liquid culture medium is what the yeast cells actually use and consists of salts and organic compounds such as glucose, potassium phosphate, ammonium chloride, and distilled water. Metal salts (such as copper sulphate) can be added to the media for mutation studies.

The agar media must be sterilized before use, and you will be supplied with sterile media for your experiment. Sterilization kills all living microorganisms in the culture media. This process is done in an autoclave which cooks the media in a steam bath at 120°C for 15 minutes.

Copper-Tolerant Mutants

You will use the replica-plating technique to test when copper-tolerant mutants in yeast arise. The feature of replica-plating which is particularly important in this test, is the ability to make several replicas of the original plate. How can we use the patterns of mutants which grow on the copper replica plates to test for the type of mutants?

1. Pre-existing mutants: If the mutants already existed on the normal plate, then mutant cells will be transferred onto the replica plates.
 Will the location of mutant colonies on the replica plates be (a) exactly the same or (b) completely different?
2. Induced mutants: If the mutants were induced to occur only after cells were transferred to the copper plates, then mutants are not being transferred from the primary plate.
 Will the location of mutant colonies on the replica plates be (a) exactly the same or (b) completely different?

Part A: Prepare Yeast Culture

Materials

You will work independently. Obtain the following:

Sterile petri plate	Ethanol solution, 70% (1 per bench)
Spreader	“To deliver” pipet, 10 ml (1 per pair)
Sterile pasteur pipet	Physiological saline solution
Sterile 20-ml vials (for dilution) (3)	Propipetter (1 per bench)
Baker's yeast (1 packet per bench)	
Autoclaved agar medium (1 litre per class)	

Prepare Plate and Pour Agar

1. Clean the top of your lab bench with 70% ethanol solution to remove dust and contaminants.
2. Label a sterile petri plate with your name, date, and the word “primary” to identify it as the original culture medium. Label the bottom of the petri plate as well as the lid.
3. Obtain a flask of agar medium. It is incubated in a water bath at 40°C to keep it liquified. This medium is “normal” and has no added copper.
4. Flame the lip of the flask containing the agar medium, to kill any contaminants (see Figure 15.1).
5. Open the lid of the sterile petri plate just enough to pour in the agar. This reduces airborne contamination.
6. Pour agar to half fill the petri plate (about 20 ml of agar). Gently swirl the agar to eliminate bubbles and to make sure the plate is entirely covered.
7. Close the lid immediately and let the agar cool for about 20 minutes so that it becomes solid.

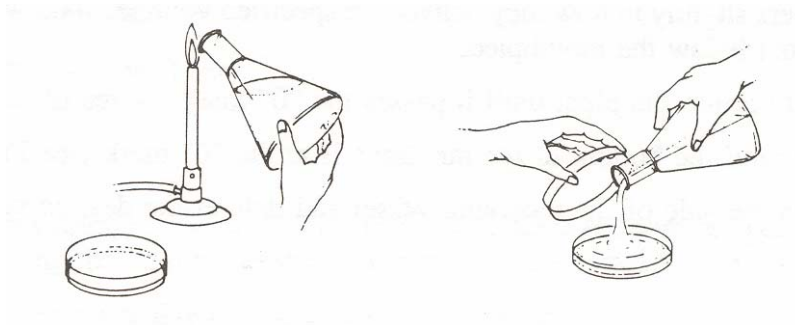


Figure 15.1. Steps in pouring an agar plate.

Prepare Yeast Suspension

Yeast cells are tiny (about 2 μm), so there is a potential for very crowded cultures — this is not good, since we want to be able to recognize individual colonies on the plates. For this reason, we suggest that you prepare a dilute suspension of yeast in the following way:

1. Weigh out 0.1 g of the baker's yeast powder on a piece of weighing paper.
2. Add the yeast to 9.9 ml of physiological saline solution (0.85% NaCl solution) in a 20 ml vial. This gives you a 1% suspension. We estimate that at this concentration, there are about 10 million cells in one drop (0.05 ml) of suspension. This is far too many cells for our purposes, so we must dilute the suspension in order to obtain a concentration of about 100,000 cells per drop.
3. Make a serial dilution to obtain a 0.01% suspension (see the text below on how to make a serial dilution, and on using a pipet).

Serial Dilutions

Let's say you have a solution (the “stock” solution) which you need to dilute before you can use it in your experiment. You may have to make a 1:100 dilution (also called a dilution factor of 100) which will result in a 1% solution. You could make your dilute solution in one step (a *single* dilution series) by adding 1 part stock solution to 99 parts water (the “dilutant”). But if you only wanted a small amount of dilute solution (say, 1 ml), you would need very precise (and expensive) equipment, since you would have to be able to accurately measure out 0.01 ml of stock and 0.99 ml of dilutant.

A serial dilution is an alternative way to obtain your dilute solution. In this example, you could make two 1:10 dilutions, as follows:

1 part stock + 9 parts dilutant = 1:10 solution (solution A)

1 part A + 9 parts dilutant = 1:100 solution (1% solution)

Using a Pipet

You will use a pipet to measure out the quantities in your dilutions. There are three basic types of pipets. Each differs slightly in how they deliver the specified volume. You will use a “to deliver” pipet; “TD” is written below the mouthpiece.

1. Draw the liquid up into the pipet until it passes the “0” mark. Wipe off the tip with a tissue.
2. Drain the pipet until the bottom of the meniscus is at the “0” mark (see Figure 15.2).
3. Touch the tip to the side of the receiving vessel and deliver the desired volume of liquid into the vessel.

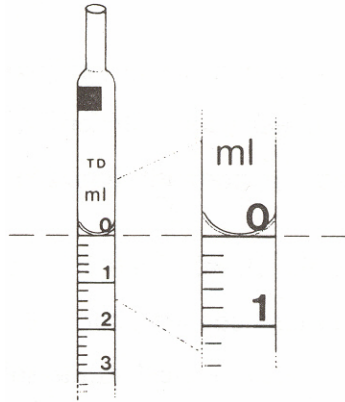


Figure 15.2. Reading a pipet.

Spread Culture

1. Swirl the dilute yeast sample to evenly distribute the cells.
2. Draw up some yeast suspension using a sterile pasteur pipet.
3. Open the petri plate, and holding the tip of the pipet about 1 cm above the centre of the plate, gently squeeze the bulb so that *1 or 2 drops* fall on the surface.
4. Swab the spreader with ethanol and air dry.
5. Spread the drop evenly over the agar surface by holding the spreader and gently rotating the petri plate. Try to distribute the yeast cells as evenly as you can.
6. Close the petri plate lid and secure it with two small pieces of masking tape.
7. Leave the petri plate sit upright for 30 minutes before moving it. This allows the yeast cells to adhere to the agar surface.
8. Take your culture home with you.

At Home

1. Keep your cultures *upside down* in a uniformly warm place (a room temperature of 20–25°C is acceptable). Storing the cultures upside down reduces evaporative water loss from the media.
2. Check your cultures every day (twice a day is even better).
3. Put your cultures in a refrigerator (but not a freezer) once the cells have multiplied to form small white colonies (about 1–2 mm in diameter) on the plate. Do not let your colonies get so large that they coalesce into a uniform mass or “lawn”. Refrigeration stops further colony growth but does not kill the cells.

Questions

1. What is agar, and what is its function in a culture medium?
2. How is the agar medium sterilized, and why is it sterilized?
3. Where are yeasts, such as *S. cerevisiae*, normally found in nature? How have humans used this type of yeast?
4. What dilution would you use to make 10 ml of a 0.1% solution from 1 litre of a 10% solution?
5. Describe two ways that you minimized contamination of your culture.
6. Why did you suspend the yeast in physiological saline solution and not, for example, distilled water?

Part B: Replica Plating

Materials

Each student will need the following:

Their “primary” plate

Sterile petri plates (2)

Sterile velvet square

Agar media containing 3 μ M of copper sulphate (CuSO_4) (1 litre per class)

Pour Copper Agar Plates

1. Label two new sterile petri plates with “+Cu” to indicate they will hold copper-containing media.
2. On the bottom rim of each plate, make a vertical mark. The mark will be used later as a guide during the replica plating.
3. Using the sterile techniques you learned in the last lab, prepare two copper plates.
4. Let the +Cu plates cool undisturbed for at least 20 minutes (copper agar can be a little sloppy compared to normal agar, so check after this time that the agar is firm).

Assemble Replica Plater

5. Clean the top of your bench with 70% ethanol solution to prevent the velvet from becoming contaminated.
6. Assemble the components of the replica plater. Obtain a piece of sterile velvet (handle carefully to keep it clean), a replica plater, and the retaining ring (which holds velvet to plater).
7. Put the ring on the clean portion of the bench, and arrange the velvet (nap side down) on top. Then, push the replica plater down onto the velvet until the retaining ring secures it to the plater.

Lift out the assembled replica plater and check that the velvet surface is flat and *slightly raised* above the ring (Figure 15.3).

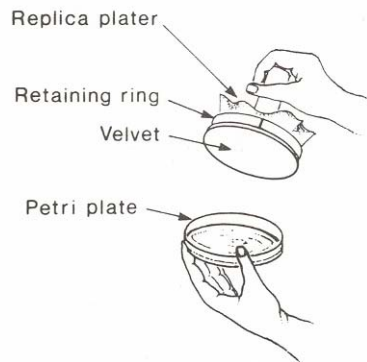


Figure 15.3. Assembled replica plater.

Make Replica 1

8. Make a vertical line on the retaining ring of the replica-plater (to assist you when you make the replicas).
9. Press the velvet surface gently down onto the primary culture so that it picks up cells from all colonies. Lift it off.
10. Align the vertical marks on the replica plater and the side of the first replica plate and gently press the velvet surface evenly onto the +Cu plate. This will transfer cells to the first copper replica plate. Close the lid immediately.

Make Replica 2

11. Repeat step 10 for the second replica plate.

At Home

12. Store the replica plates at home. Mutant colonies will not grow as fast on the copper plates. It may take 1 week to see good colonies.
13. *Refrigerate* your cultures once the colonies have grown to about 3 mm in diameter.

Questions

1. Why did you store your culture upside down?
2. Briefly describe the main steps in replica-plating.
3. Why was copper added to the agar media for the replica plates?

4. Describe two potential mistakes during the replica-plating procedure which could negatively influence your experimental results.
5. Based on the growth on your replica plates, how common are copper-tolerant mutants in the yeast population?

Part C: Analysis

Compare Replica Patterns

1. Position both replica plates so the vertical marks are in the same position. Examine the colonies on the two +Cu plates.
2. Draw the positions of the mutant colonies on each replicate plate in the templates provided in Figure 15.4. Mutant colonies are the large colonies. These should not be confused with the “background” growth of normal cells which can sometimes survive the copper, but tend to grow very slowly.
3. From the spatial arrangement of mutant colonies how do you answer the original question: When do adaptive mutants arise in a population?

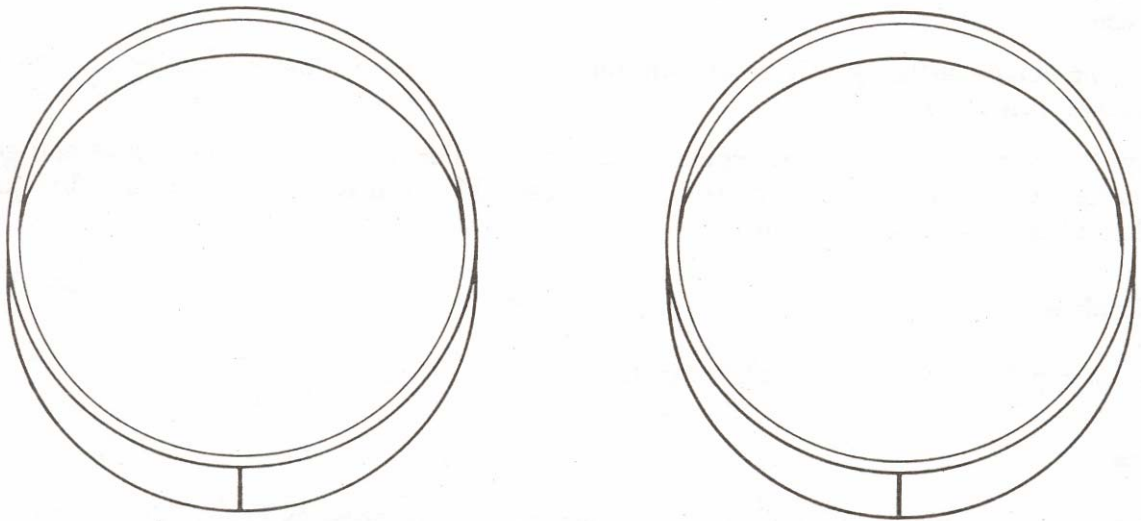


Figure 15.4. Petri plate templates. Draw in the position of colonies on each replica plate.

Review Questions

1. Why are mutants important in a population?
2. What is the difference between a spontaneous mutation and an induced mutation?
3. How does the replica-plating technique allow us to test for the timing of adaptive mutations?
4. Draw the expected pattern of mutant colonies on replica copper plates if (a) copper tolerant mutants were pre-existing in the yeast population, and (b) mutants were induced by exposure to copper.

5. A researcher studying the timing of mutations, finds some *but not all*, mutant colonies match on two replica plates. What can she conclude from her experiment, and why?

Notes for the Instructor

Part A: Prepare Yeast Culture

To introduce the exercise, the instructor can initiate a class discussion of what mutation is, and what spontaneous and induced mutations are. Spontaneous mutations arise at any time, and randomly with respect to traits. Induced mutations arise in the appropriate traits when required; that is, the environmental challenge directs the occurrence and site of the mutation.

While this exercise was developed from an evolutionary perspective, there is clearly scope for much more rigorous emphasis on the microbiological aspects such as sterile technique. We have students make a suspension of yeast from commercially-available bakers yeast, in part for the novelty value. One packet of yeast goes a long way. We suggest preparing a 0.01% suspension to ensure that 1–2 drops will contain a few mutants. The suspension must be prepared by serial dilution—this gives the students an opportunity to learn about pipets and dilutions. The following series can be used:

Add 0.1 g to 9.9 ml 0.85% saline solution* = 1% suspension

Add 1 ml 1% suspension to 9 ml saline = 0.1% suspension

Add 1 ml 0.1% suspension to 9 ml saline = 0.01% suspension

* Distilled water can be substituted for saline solution for short-term cultures.

When spreading the suspension on the agar, have the students spread the drop as evenly and completely so that colonies are well spaced eventually.

Part B: Replica Plating

The copper agar tends to be a bit sloppier than just the regular agar media. Allow it to sit for at least 20 minutes to solidify. When students assemble the replica plater, they must ensure that the velvet is held perfectly flat by the retaining ring, and that the retaining ring is slightly below the velvet surface. Don't contact the velvet on the culture too long or too firmly, or it will lift off agar and colonies.

Part C: Analysis

Encourage students to diligently draw the positions of the main colonies on each replica plate. Mutant yeast cells will grow normally on the copper plates, whereas normal (wild-type) cells will have very poor growth. As a consequence, you should see two types of colonies growing on the replicas. However, it is the position of the larger (mutant) colonies which students should draw on the diagrams.

From results obtained over a 2-year period, there is good spatial correspondence between mutant colonies on each plate which supports the spontaneous mutation hypothesis. This does not mean, however, that there is perfect match — often the second plate is a partial replica because of the incomplete transfer of cells.

Suggested Answers to Questions*Part A: Prepare Yeast Culture*

1. What is agar, and what is its function in a culture medium?
 - Agar is a polysaccharide derived from red seaweeds, and it functions to provide a firm substrate for culturing yeast.
2. How is the agar medium sterilized, and why is it sterilized?
 - Agar is sterilized by the autoclave method in a steam bath. Sterilization reduces the chance of other, unwanted microorganisms growing on the cultures.
3. Where are yeasts, such as *S. cerevisiae*, normally found in nature? How have humans used this type of yeast?
 - Yeasts such as *S. cerevisiae* are naturally found on plant surfaces which have sugars such as fruits and some leaves. This yeast is used by bakers, brewers, and distillers. In the brewing industry, alcohol is produced by the fermentation of molasses (sugar) by the yeast. The yeast convert about 90% of the sugar into alcohol and CO₂.
4. What dilution would you use to make 10 ml of a 1% solution from 1 litre of a 10% solution?
 - A 1:10 dilution is needed, and you would use 1 part stock + 9 parts dilutant. In this case, since only 10 ml is required, 1 ml stock + 9 ml distilled water would be sufficient.
5. Describe two ways that you minimised contamination of your cultures.
 - Contamination was reduced by (a) cleaning down the bench, (b) use of sterile petri dishes, (c) flaming the mouth of agar flask, (d) when pouring the plates, keeping lid off for as short a time as possible, and (e) used a sterile spreader.
6. Why did you suspend the yeast in physiological saline solution and not, for example, distilled water?
 - Yeast cultures in suspension are best in a saline solution which approximates the concentration inside the cell.

Part B: Replica Plating

1. Why did you store your culture upside down?
 - Storage of cultures upside down reduces evaporative loss of moisture from the medium.
2. Briefly describe the main steps in replica-plating.
 - Assemble replica plater with a sterile velvet. Make vertical line on each copper plate. There is a vertical line on the edge of the replica plater as well. Press the plater into the primary plate so as to pick up as many colonies as possible. Align vertical marks and press plater into each copper plate.

3. Why was copper added to the agar media for the replica plates?
 - In this exercise, copper is added to the media to make a hostile environment with which to expose copper-tolerant mutants. A range of chemicals not normally encountered in high concentrations by yeast populations can be used.
4. Describe two potential mistakes during the replica-plating procedure which could negatively influence your experimental results.
 - (a) The velvet is not even and tightly held in the plater, resulting in uneven plating, (b) the velvet surface does not contact the surface of the plates well so that only a partial pattern is transferred, and (c) vertical markers are not aligned for each replica plate.
5. Based on the growth on your replica plates, how common are copper-tolerant mutants in the yeast population?
 - There should be about 1 mutant cell per 100,000 cells. Expect about 0–10 mutant colonies per copper plate.

Review Questions

1. Why are mutants important in a population?
 - Mutants represent the pool of variants in a population which, while not necessarily ideally suited to current conditions, may be beneficial in the future if the environment is altered.
2. What is the difference between a spontaneous mutation and an induced mutation?
 - Spontaneous mutations occur through random errors in the transcription process, and thus can occur at any time, under any conditions. Induced mutations are directly caused by mutagens such as radiation or chemicals, and they occur at the time of mutagen action.
3. How does the replica-plating allow us to test for the timing of adaptive mutants?
 - Replica-plating allows replicate plates with the exact same spatial arrangement to be set up. Any colonies which subsequently develop on the copper plates, are mutants. The basic assumption is that if mutants are induced by exposure to copper, then the chances are extremely small that the same cells on both replicates will be induced to mutate. A match of the spatial pattern of mutant colonies on each replica plate more than likely means the mutant cells pre-existed on the primary plate.
4. Draw the expected pattern of mutant colonies on replica copper plates if (a) copper-tolerant mutants were preexisting in the yeast population, and (b) mutants were induced by exposure to copper.
 - See Figure 15.5.
5. A researcher studying the timing of mutations, finds some but not all, mutant colonies match on two replica plates. What can she conclude from her experiment, and why?
 - The match of at least some colonies suggests spontaneous mutations. All colonies may not match if there was partial transfer of cells onto one of the replica plates.

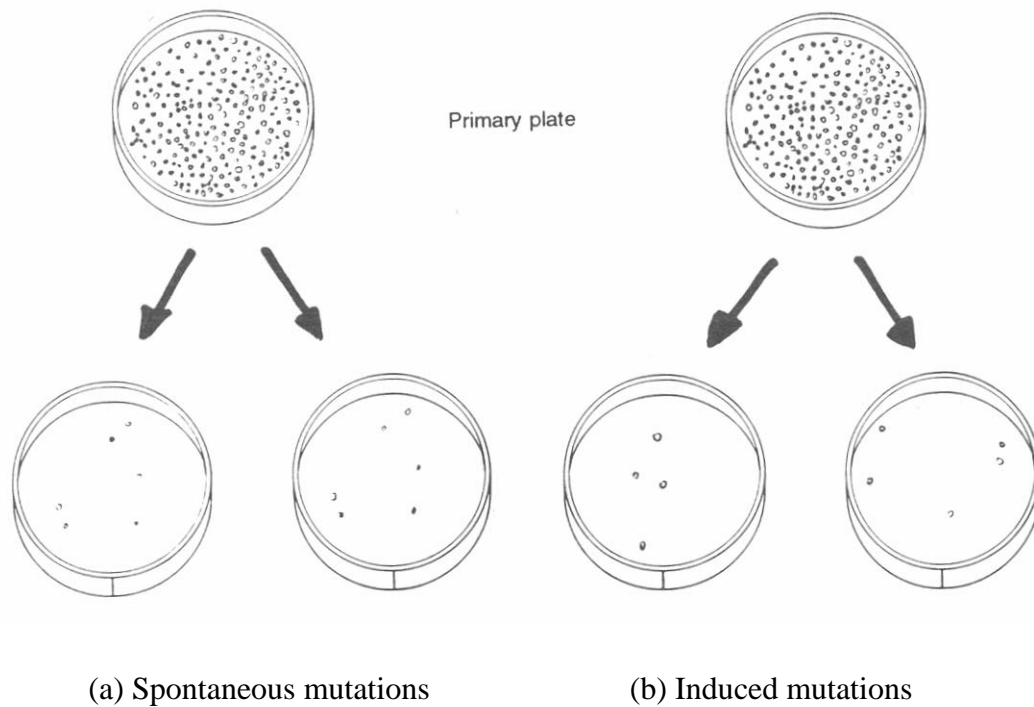


Figure 15.5. Patterns of mutant colonies on replica plates for the two types of mutations.

Acknowledgements

Thanks to Hemant Chikarme, Andy Rubaszek, Marianna Puszynska, and Dominic Fenech for help and advise on technical and conceptual components of this exercise. Thanks also to participants in the ABLE workshop for their feedback on all aspects of the exercise.

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Purves, W. K., G. H. Orians, and H. C. Heller. 1992. *Life: The science of biology*. Sinauer Associates, Sunderland, Massachusetts, 1145 pages.

APPENDIX A
Building a Replica Plater

All components of the replica plater (Figure 15.6) are made of plexiglass acrylic. The handle is made of 1/8" tubing, is 1.5" long, and is "glued" to the disk with an acrylic solvent. The disk is 7.5 mm thick and 3.000" in diameter. The ring is 2.6 mm thick and 7.5 mm high, with an inside diameter of 3.016" and a camfered edge.

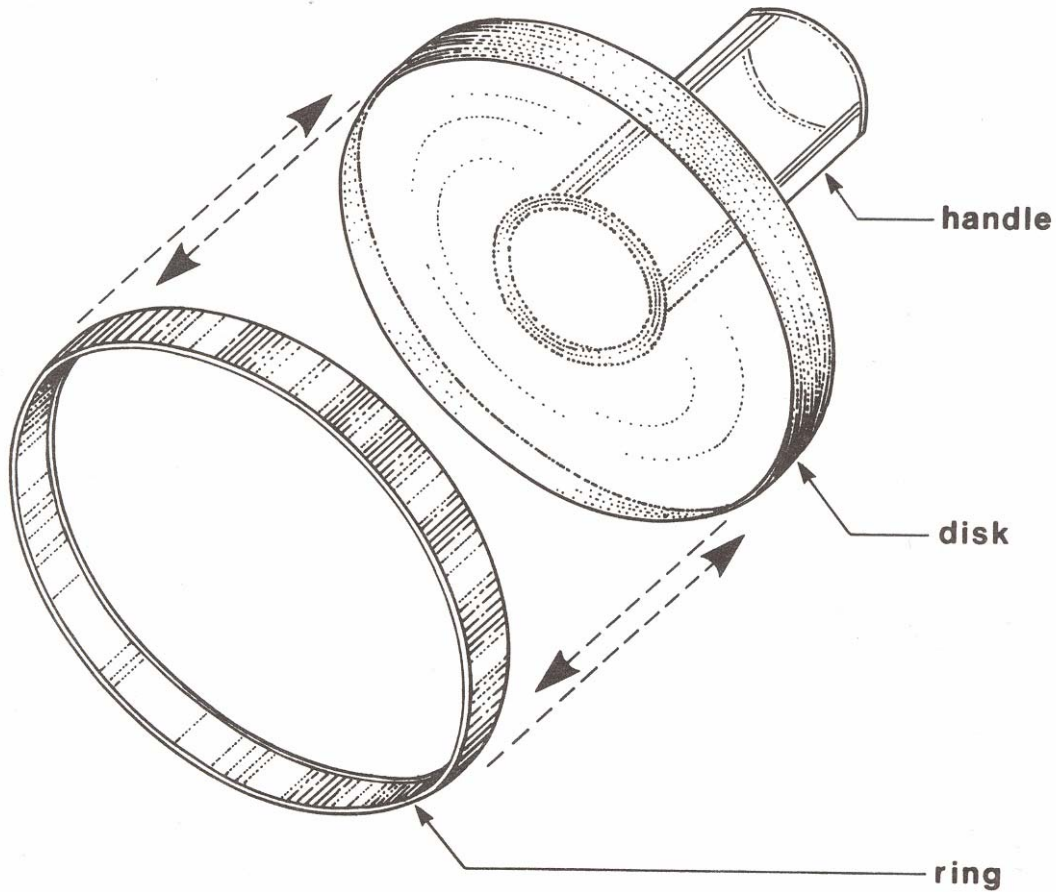


Figure 15.6. Replica plater (actual size).