NATIONAL CENTRE FOR BIOTECHNOLOGY EDUCATION

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Making and testing an antibiotic

Production of streptomycin by *Streptomyces griseus*

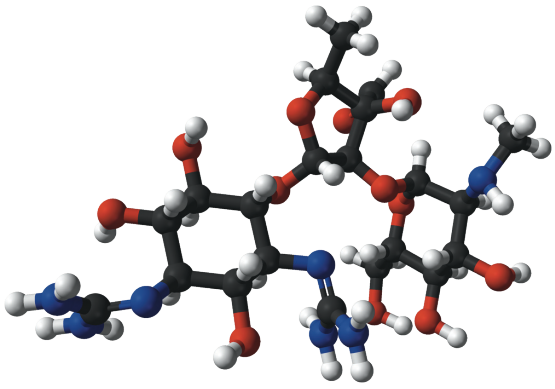
Aim

This practical protocol tests the effectiveness of the antibiotic streptomycin against several different species of microorganisms.

Introduction

Almost all antibiotics are produced by microorganisms. They work in variety of ways to inhibit the growth of, or kill, microorganisms. Streptomycin was one of the first antibiotics to be used medically: it was the first effective cure for tuberculosis (TB).

Streptomycin is produced by some strains of the bacterium *Streptomyces griseus*, which release the antibiotic into the surrounding growth medium. It was discovered in 1943 by Albert Schatz, who was then a 23-year-old research student at Rutgers University in the USA. Unfortunately, Schatz was taken advantage of by his supervisor Selman Waksman, who increasingly claimed credit for the discovery. Schatz successfully sued Waksman in 1950, but this action damaged his career prospects and he was never able to find work in microbiology at a high level. In 1952, Waksman was awarded the Nobel Prize, for 'his' discovery, and Schatz was forgotten. It was not until 1994 that Rutgers University finally recognised Schatz's achievement and awarded him the Rutgers medal, the university's highest accolade. In 2010, five years after Schatz's death, his laboratory notebooks were found at Rutgers, confirming his account of the discovery.



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Structure of Streptomycin.

How does streptomycin work?

Streptomycin inhibits protein synthesis in bacteria. It binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit.

This leads to codon misreading, eventual inhibition of protein synthesis and ultimately death of microbial cells through mechanisms that are still not understood.

Humans have ribosomes which are structurally different from those in bacteria, so the drug does not have this effect in human cells. At low concentrations, however, streptomycin only inhibits growth of the bacteria by inducing prokaryotic ribosomes to misread mRNA.

Streptomycin has been superceded by other antibiotics for the treatment of tuberculosis. Drug-resistant strains of the causative bacterium (Mycobacterium tuberculosis) are increasingly common. Today about half of the antibiotics in use come from actinomcyetes (the group that Streptomyces is classified in).

Safety guidelines

Good microbiology laboratory practice must be followed when carrying out this work.

Equipment and materials

Needed by each person or group

Equipment
- Wire inoculating loop
- Bunsen burner
- Fine-tipped permanent marker pen
- Ruler

Materials
- Petri dish with a streak of *Streptomyces griseus* (labelled: **S**)
- Paper towels

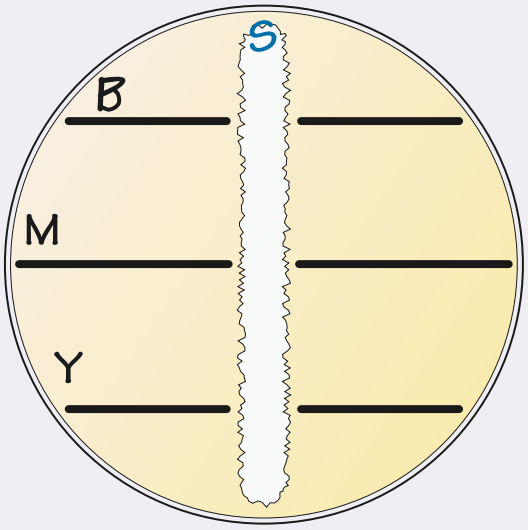
In addition, you should have access to Petri dishes of three microbial cultures (which may be shared with other students):
- *Micrococcus roseus*, labelled: **M**
- *Bacillus subtilis*, labelled: **B**,
- *Saccharomyces carlsbergensis* (brewer's yeast), labelled: **Y**

You should also have access to:
- Disinfectant solution for wiping down the bench
- Lighter or matches
- Self-adhesive tape

Procedure

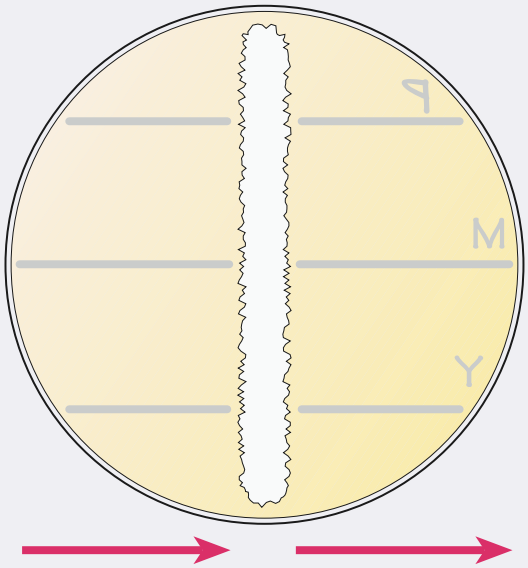
1 Without opening the Petri dish, use the ruler and marker pen to draw six lines and the letters **B**, **M** and **Y** on the BASE of the Petri dish as shown in Figure 1. *These will be the guides that help you to streak the plate later.*

Fig. 1



BASE OF PETRI DISH

Fig. 2



SURFACE OF AGAR

2 Put the plate to one side and use the paper towels and disinfectant to wipe down your work area.

3 Wash and dry your hands.

4 Obtain one of the three cultures (labelled: **B**, **M** or **Y**).

5 Light the Bunsen burner and adjust it to produce a blue flame.

6 Sterilise the inoculating loop correctly in the flame.

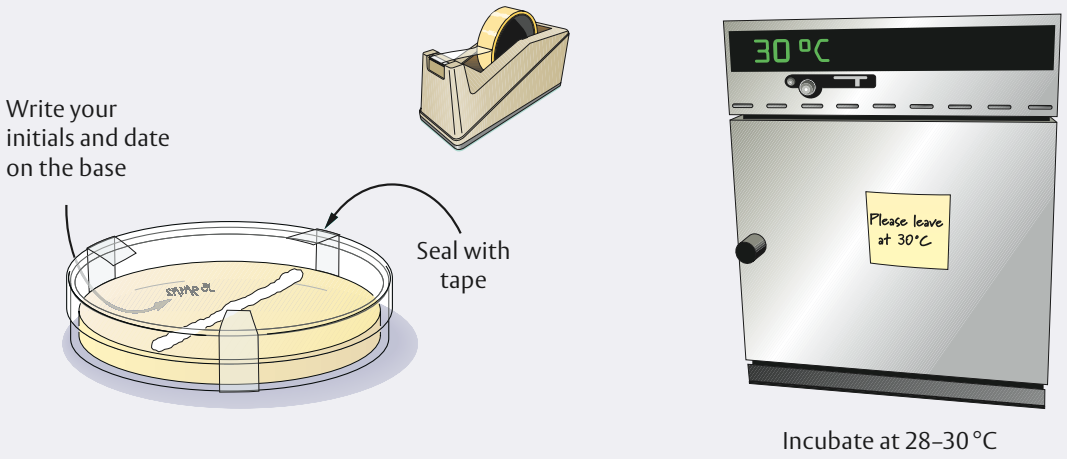
7 Using the lines on the base of the Petri dish as a guide, streak the culture, in two separate streaks, onto the agar in the correct place (B, M or Y) in the direction shown by the arrows in Figure 2. **VERY IMPORTANT: Do NOT touch the *Streptomyces* streak with the loop as you inoculate the plate.**

8 Flame the loop correctly to sterilise it again.

9 Repeat steps 5, 6 and 7 with the other two cultures, using the lines and letters you have drawn on the base of the plate as a guide as to where to streak the agar.

10 Seal the Petri dish two or three times at the edges with tape as shown in Figure 3.

Fig. 3



11 Label the base of the Petri dish with your initials and surname.

12 Incubate the plate, inverted, at 28–30 °C, for 48 hours.

13 Wipe down your work area with disinfectant.

14 Wash and dry your hands.

After incubation

1 Draw a labelled diagram of your plate. Include outlines to show all areas of microbial growth.

2 When you streaked the plate, why was it important that you did not touch the *Streptomyces*?

3 Describe your results in words as accurately as you can.

4 What can you conclude about the effect of streptomycin on the growth of the three microorganisms? With reference to how Streptomycin works, suggest reasons for your findings.

Teacher's and technician's notes

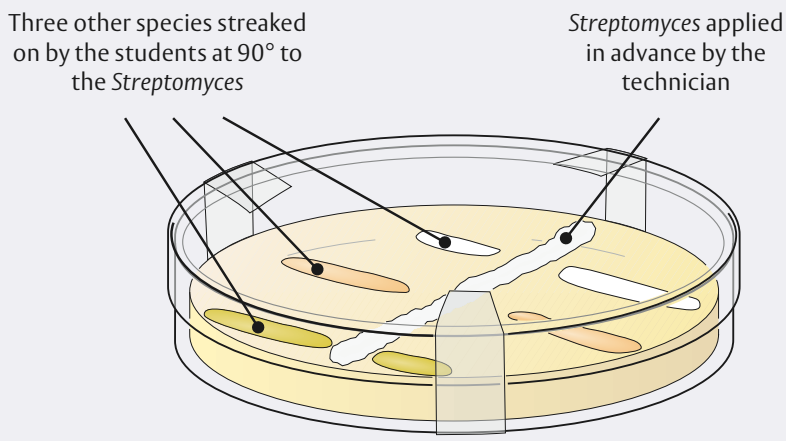
Outline of the activity

This practical activity involves the use of *Streptomcyces griseus*, a filamentous soil bacterium that produces the antibiotic streptomycin.

Students are provided with a plate that has been inoculated with a streak of *S. griseus* several days in advance, so that the bacteria are growing well and releasing streptomycin into the surrounding agar medium.

Each student inoculates their *Streptomcyes* plate with three additional microbial species at right angles to the *Streptomyces* streak (Fig. 1). One species is a yeast, the two bacterial species are both Gram-positive (other species can be used if you wish).

The plates are incubated for 48 hours, then returned to the students for examination. Students record their observations and assess which of the species they applied to the plate are affected by streptomycin.



Timing

Teachers or technicians will need to prepare plates of *Streptomyces griseus* 4–5 days before Part 1. *Streptomyces* has a more complex life cycle than most other bacteria, and takes longer to grow. Cultures of the other microorganisms may be prepared at the same time, or at least 48 hours before the first session.

Because 48 hours must be allowed for incubation of the students' inoculated plates after Part 1, this activity must be split over two sessions, namely:

- a first session for students to inoculate the plates – Part 1
- a second session, two or more days after the plates have been incubated, for students to record and analyse the results – Part 2.

Safety

Good microbiology laboratory practice

Good microbiology laboratory practice must be followed by teachers, technicians and students. Teachers and technicians should refer to the microbiology safety guidelines in: *Topics in Safety* (2001) Third Edition. Association for Science Education. ISBN: 086357 316 9. Topic 15, 'Microbiology and Biotechnology' (pages 87–98).

The relevant part of this publication and many other useful documents can be downloaded free-of-charge from the Microbiology Society's education web site: **www.microbiologyonline.org.uk/teachers/safety-information**

Please note that CLEAPSS, SSERC and other school safety organisations recommend that disposable gloves should NOT be worn for microbiological work unless there is a specific health and safety reason for this *e.g.*, if a person has cuts or abrasions on their hands or a skin condition such as eczema.

Equipment and materials

Needed by each student or group

Equipment

- Wire inoculating loop
- Bunsen burner
- Fine-tipped permanent marker pen for writing on the Petri dish
- Ruler

Materials

- Standard (90 mm diameter) Petri dish containing 15–20 cm^3 of glucose nutrient agar, that has been inoculated with *Streptomyces griseus* as described below, then incubated for 4–5 days at 28–30 °C
- Paper towels for applying disinfectant to the bench

Each student must have access to the following microorganisms, with each culture on a separate Petri dish of glucose nutrient agar:

- *Micrococcus roseus* (Gram positive), labelled: **M**
- *Bacillus subtilis,* labelled: **B** (Gram positive),
- *Saccharomyces carlsbergensis* (brewer's yeast, a eukaryote), labelled: **Y**

Note
Because the Streptomyces plates will be kept for some time, they contain slightly more growth medium than is normally used.

Each culture plate may be shared by up to three students if necessary, but should not be used for more than one practical session because of the risk of cross-contamination.

Students will also require access to:

- Freshly-prepared solution of *Virkon*® disinfectant, for disinfecting the bench
- Gas lighter or matches
- Transparent self-adhesive tape, for sealing the Petri dishes after inoculation
- Hand washing facilities

Technicians may wish to prepare a few spare sets of the materials and apparatus available in case students require replacements. They should also have a supply of waterproof plasters and disposable plastic gloves for those students who need them (see Safety note above).

In addition, the teacher or technician *only* will need:

For preparing the plates (see below)
- Wire inoculation loop
- Sterile cotton buds (prepared by autoclaving the cotton buds in a beaker loosely covered with aluminium foil — do not use cotton buds with plastic 'stems' as these will melt)
- 10 cm^3 of sterile nutrient broth in a sterile Universal bottle
- Bunsen burner
- Permanent fine-tipped marker pen for writing on the plates
- Autoclave or pressure cooker and other items for preparing agar media
- Access to an incubator set at 28–30 °C

At the end of Part 1
- Access to an incubator set at 28–30 °C

At the end of Part 2
- Autoclave or pressure cooker and autoclave bags for disposal of the used plates

Preparation

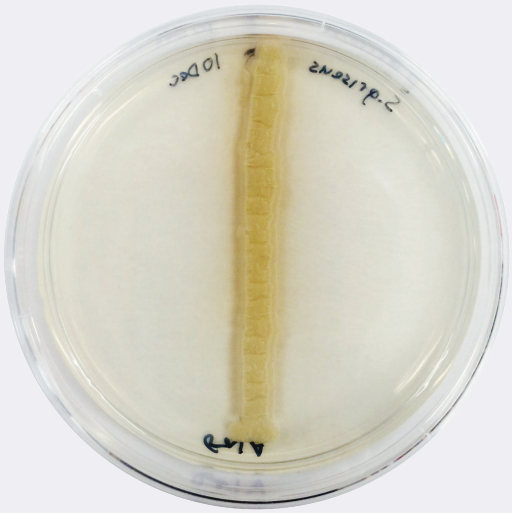
6–8 days before the first practical session (Part 1)

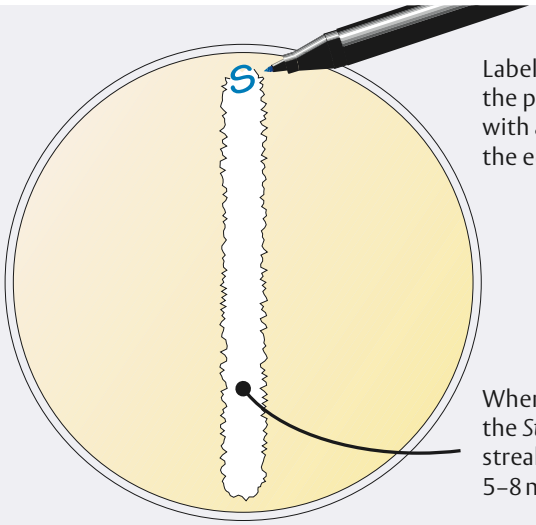
Preparation of the *Streptomyces griseus* plates

Each student will require one standard (90 mm diameter) Petri dish containing 15–20 cm^3 of sterile glucose nutrient agar (GNA) that has been inoculated with a single central streak of *Streptomyces griseus*. Because *Streptomyces* cultures can be difficult to manipulate, the easiest way to do this is is as follows:

- use a wire loop to aseptically inoculate some sterile nutrient broth with a scraping of *Streptomyces* from a slope culture, then incubate the broth for 2–3 days at 28–30 °C until the broth is cloudy;
- using a permanent marker pen and ruler, draw a straight line bisecting the base of each Petri dish of GNA;
- using the line on the base of the dish as a guide, use a sterile cotton bud to 'paint' a line of the liquid *Streptomyces* culture onto the agar medium each plate;
- incubate the plates, inverted, at 28–30 °C for 4–5 days, until the *Streptomyces* streak has grown to 6–8 mm wide.

Note: glucose nutrient agar is standard nutrient agar to which a small amount of glucose has been added, so that it may better support the growth of the yeast used in this practical (yeast does not grow well on plain nutrient agar). All microorganisms used for this practical activity should be grown on the same medium.





2–4 days before the first practical session (Part 1)

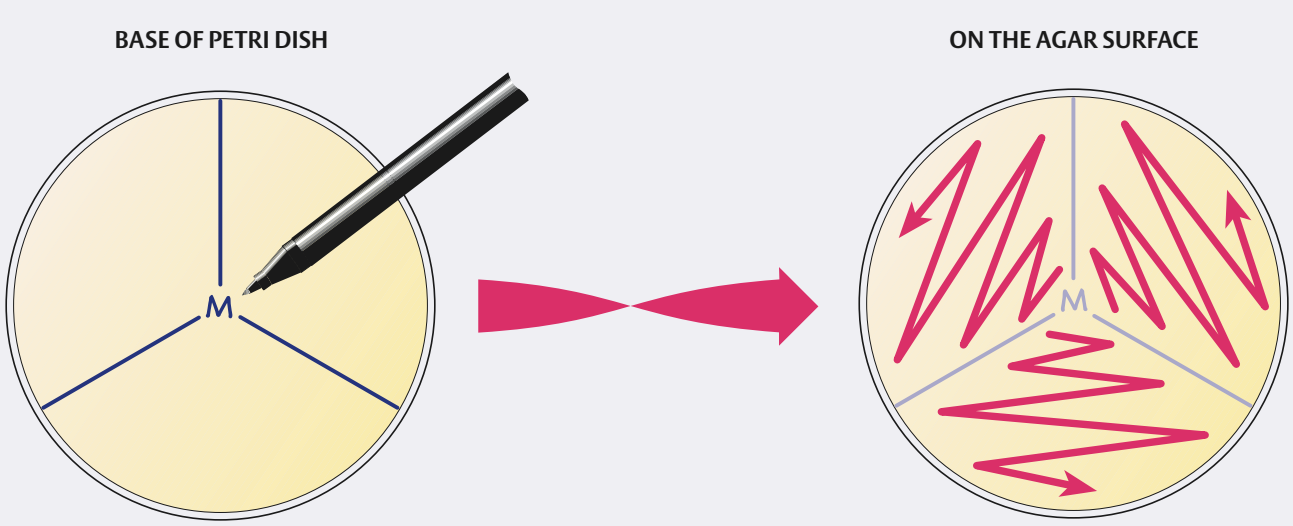
Preparation of the stock culture plates

Each student must have access to the following stock cultures (each species grown on a separate Petri dish of glucose nutrient agar):

- *Micrococcus roseus*, labelled: **M**
- *Bacillus subtilis*, labelled: **B**
- *Saccharomyces carlsbergensis*, labelled: **Y**

The plates should be inoculated as shown below, with three separate zones from which students can each take some culture.

Each plate may be shared by up to three students if necessary, but should not be used for more than one practical session because of the risk of cross-contamination.



At the end of the first practical session (Part 1)

Incubate the students' plates, agar medium uppermost, at 28–30 °C for at least 48 hours.

After incubation, if the plates are not to be used immediately by the students, they can be stored, with agar medium uppermost, in a fridge at 3–5 °C before Part 2.

IMPORTANT: The plates should be removed from the fridge a few hours before Part 2, to allow any condensation in the Petri dishes to evaporate. If the plates have been stored inverted in the fridge, the amount of condensation should be minimal.

At the end of the second practical session (Part 2)

All of the plates must be disposed of by autoclaving.

Suppliers

The NCBE provides a pack of the four microbial cultures needed for this practical activity. See: **www.ncbe.reading.ac.uk**

Additional information

Finding Dr. Schatz: The discovery of Streptomycin and a life it saved by Inge Auerbacher (2006) iUniverse, Inc. ISBN: 978 0595379972. *Written by a medical researcher who, as a girl, had contracted TB in a Nazi concentration camp, who later moved to the USA and was cured with Streptomycin. Later in life she contacted the by then infirm Albert Schatz and co-wrote this book with him. It was published after his death in 2005 and is as close as he came to writing a biography.*

Notebooks shed light on an antibiotic's contested discovery by Peter Pringle (11 June, 2012) *The New York Times* **http://www.nytimes.com/2012/06/12/science/notebooks-shed-light-on-an-antibiotic-discovery-and-a-mentors-betrayal.html?_r=0** *Account by Peter Pringle of the rediscovery of Albert Schatz's laboratory notebooks at Rutgers University in 2010 (after Schatz had died) confirming that his account of the discovery of Streptomycin was true.*

Time the great healer by Veronique Mistiaen (2 December 2012) *The Guardian* **http://www.theguardian.com/education/2002/nov/02/research.highereducation** *This article describes how Albert Schatz's role in the discovery of streptomycin was overlooked for decades while Selman Waksman gained all the glory, a great deal of money and a Nobel prize.*

Experiment Eleven: Deceit and betrayal in the discovery of the cure for tuberculosis by Peter Pringle (2013) Bloomsbury. ISBN: 978 14088 31069. *A thoroughly-researched and well-written account of the discovery of Streptomycin and the conflict between Albert Schatz and Selman Waksman.*

How does Streptomycin work? by Laurence A. Moran **http://sandwalk.blogspot.co.uk/2009/03/how-does-streptomycin-work.html**

Molecule of the month: Aminoglycoside antibiotics. **http://pdb101.rcsb.org/motm/146**

APPENDIX I

Preparing glucose nutrient agar plates

The amount of nutrient agar powder required varies according the make/brand. Approximately 14 g of powder is usually required to make 500 cm^3 of medium, which will be sufficient for 35–40 plates.

1 Add the amount of nutrient agar powder required to make 500 cm^3 of medium to a clean 1 dm^3 (litre) conical flask that has been rinsed with distilled or deionised water.

2 Add 5 g of glucose powder to the flask.

3 Add 500 cm^3 of distilled or deionised water to the flask and stir well.

4 Adjust the pH to 7.5 using 1M NaOH and a pH meter or pH paper (typically, you might need to add about 1.0 cm^3 of 1M NaOH to adjust the pH). The pH may be re-adjusted with 1M HCl if necessary.

5 Cover the mouth of the flask with aluminium foil. Autoclave (or use a pressure cooker) to sterilise the medium for 15–20 minutes at 121 °C.

6 After autoclaving, let the flask cool to 50–55 °C (until the flask can be held comfortably in your hands). *Note: The flask of medium should be kept molten by standing it in a water bath at 50 °C. If the agar medium begins to solidify, it can be re-melted by autoclaving it for not more than 5 minutes.*

7 While the agar medium is cooling, open the bag of sterile Petri dishes. Cut the end of the plastic bag carefully so that it can be re-used to store the poured plates. Spread the Petri dishes out on the bench, unopened, ready to pour the agar.

8 Swirl the flask carefully to mix the agar evenly after autoclaving. (When the mixture was autoclaved, the agar may have sunk to the bottom of the flask.)

9 When the agar has cooled to 50–55 °C, lift the lid of each Petri dish just enough to pour the agar in. Do not put the Petri dish lid down on the bench. Quickly add enough agar to cover the bottom of the plate (you will need between 12 and 15 cm^3 per Petri dish). Replace the lid immediately and tilt the plate to spread the agar.

10 Continue pouring plates. Occasionally, flame the mouth of the flask to maintain sterility.

11 If necessary, remove bubbles from the surface of the poured agar by briefly touching the surface with a Bunsen burner flame while the agar is still molten.

12 Allow the agar to solidify, undisturbed (this takes about 15 minutes).

13 Stack the plates, with the agar medium uppermost, in their original plastic sleeves for storage. The plates should be stored at room temperature in a dark cupboard (light can degrade the nutrients in agar). Storing the plates in a fridge might lead to excessive condensation.

Plates that are contaminated MUST NOT be used. They should be disposed of by autoclaving.