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Antibiotic resistance in *Escherichia coli*

A practical investigation of bacterial conjugation

Aim

In this practical protocol students investigate the way in which bacteria acquire antibiotic resistance through conjugation.

Introduction

Shortly after the use of antibiotics became widespread in the 1940s, strains of bacteria began to emerge that were resistant to them. Selection pressures caused by the inappropriate or careless use of antibiotics tend to increase the prevalence of resistant populations of microorganisms; hence current concerns about hospital-acquired infections, a heightened awareness of the need for cleanliness and attempts to more strictly control the use of antibiotics. Antibiotic resistance is one of the few modern examples of evolution to be found in current school textbooks. The usual examples cited are methicillin-resistant *Staphylococcus aureus* (MRSA) and strains of *Mycobacterium tuberculosis* (the usual cause of tuberculosis) that are resistant to antibiotic treatment (the latter is often treated with rifampicin).

Charles Darwin, of course, knew nothing about this: the nature of disease was a mystery to him for most of his life. He worried that his own marriage to his cousin, Emma Wedgwood, might be the cause of a 'hereditary weakness' that had contributed to the death of his beloved daughter Annie. So concerned was he that he tried to have a question on the topic of first cousin marriages included in the National Census of 1871, but the idea was turned down by parliament as an unwarranted breach of privacy. Darwin made his anger clear in the *Descent of Man*, where he fumed that 'ignorant members of our legislature' had prevented an important, potentially life-saving scientific enquiry.

In 1877, as the germ theory of disease was being developed, Ferdinand Cohn, a friend at the University of Breslau (now Wrocław in Poland) sent

Darwin one of the first photographs of a micro-organism. Charles replied:

"I well remember saying to myself between twenty and thirty years ago, that if ever the origin of any infectious disease could be proved, it would be the greatest triumph to Science; and now I rejoice to have seen that triumph".

It was twenty-six years since Annie had died. The photograph had been taken by the brilliant pioneering German microbiologist Robert Koch (Koch's assistant Julius Petri, invented the Petri dish). Koch identified the microbe that causes tuberculosis five years later, less than a month before Darwin died. For his work on tuberculosis, Koch received a Nobel Prize in 1905. The evidence available today suggests that Annie Darwin had died from that dreaded disease [1].



▲ Annie Darwin, who died at the age of ten, probably from tuberculosis.

Darwin assumed that hereditary characteristics were always passed from one generation to the next, 'vertically'. He also thought that evolutionary change occurred only slowly and by the gradual accumulation of mutations. This is often the case with microorganisms — genes conferring resistance to antibiotics are sometimes located on the bacterial chromosome as in, for example, the antibiotic resistance in MRSA. Recent research has cast light upon how such resistance can arise and be maintained through the selective pressure of antibiotic treatment in a medical context [2]. Genes that remain in the bacterial chromosome are transferred vertically ('down the generations') and not between bacterial strains or species. Often, however, microbial genes are transferred 'horizontally', that is, from one bacterial strain or species to another.

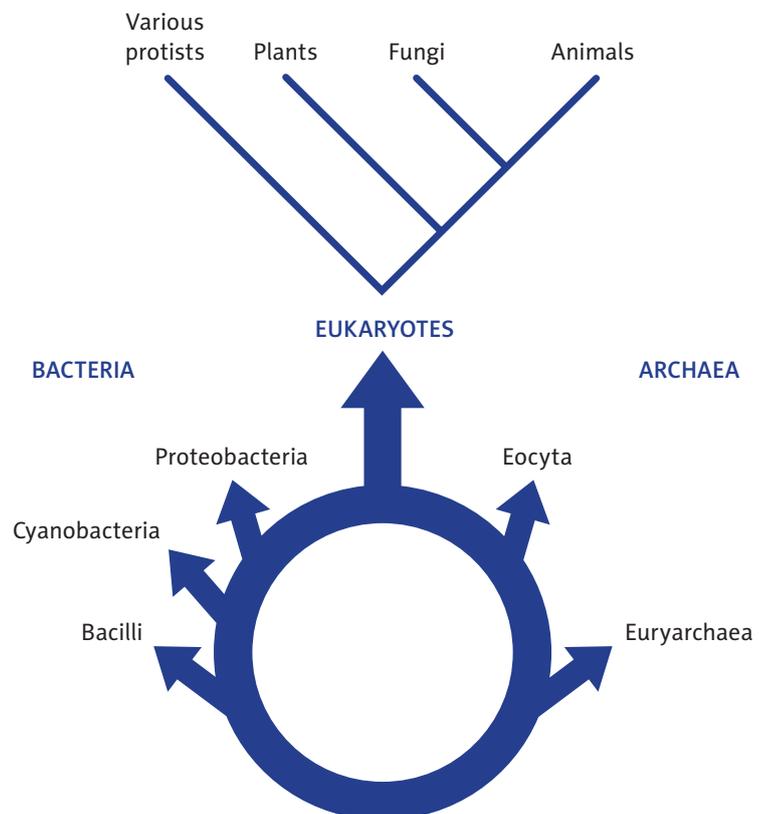
There are three principal modes of horizontal gene transfer: transformation, in which 'naked' DNA is picked up by cells; transduction, in which the transfer is mediated by a virus and conjugation, in which DNA is transferred between cells via a special 'sex pilus' or tube. Transformation is probably the most common mechanism of horizontal gene transfer in nature; many bacteria are naturally 'competent', that is, able to pick up DNA from their environment. However, the fate of this DNA once it has entered the cell varies — often

it is degraded by enzymes or is not functional. In contrast, transduction is a central, if not the most important, gene transfer mechanism in the generation of genomic diversity and bacterial evolution. Conjugation is also relatively common. Here the DNA is transferred in the form of a plasmid. Plasmids are small independently-replicating rings of DNA, typically carrying a handful of non-essential genes — these are often genes which impart the ability to withstand antibiotic action. Conjugation is therefore an important means by which antibiotic resistance is spread between bacteria. The transferred genes may subsequently be incorporated into the bacterial chromosome, as has happened for instance with resistance to antibiotics such as tetracycline and streptomycin.

Plasmid-mediated evolution is fast because whole functional 'modules' are lost and gained, rather than the gradual process of mutation and selection that is more commonly encountered in the chromosomal DNA [3]. These are mechanisms of evolution that Charles Darwin did not anticipate.

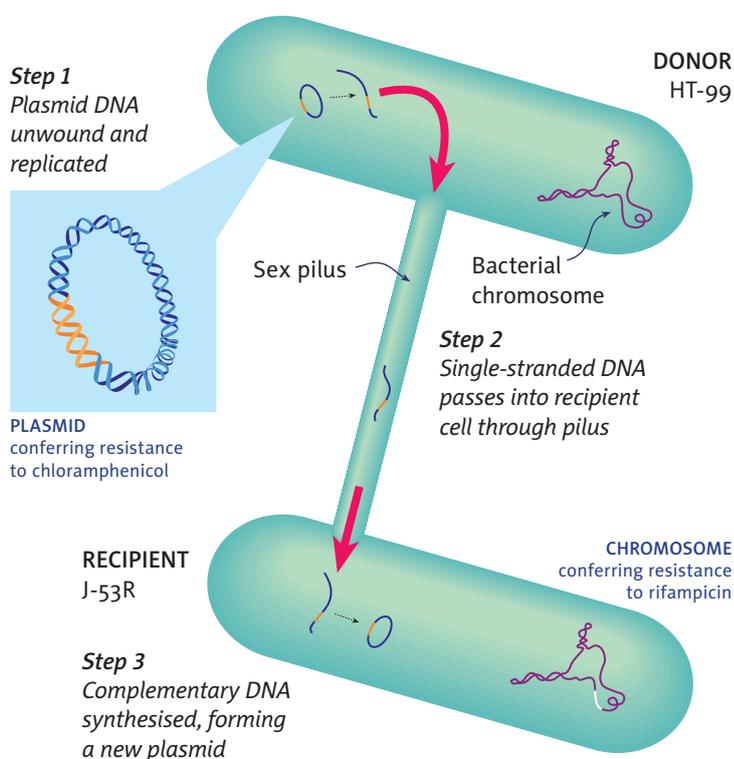
Microbial evolution is now known to be very different from the tree-like, branching phylogeny that was proposed by Darwin [4]. Studies of horizontal gene transfer provide evidence for the exchange of genetic information within bacterial species and also between distant taxonomic groups and even across kingdom borders [5, 6].

*A modern view of the 'tree of life'. The DNA evidence suggests that ancient archaea fused with bacteria, giving rise to the eukaryotes. The base of the tree is a ring, since microorganisms can exchange their DNA. Adapted from Rivera, M. and Lake, J. (2004) The ring of life provides evidence for a genome fusion origin of eukaryotes. *Nature* 431: 152–155. [6]*



Outline of the practical activity

In this practical protocol students investigate one way in which bacteria acquire antibiotic resistance through conjugation. The recipient strain *Escherichia coli* J-53R carries on its chromosome a gene conferring resistance to the antibiotic rifampicin. The donor strain, *E. coli* HT-99, harbours a plasmid that includes a gene conferring resistance to a second antibiotic, chloramphenicol. Liquid cultures of the two strains of bacteria are 'mated'. The recipient, donor and 'mated' cells are then plated on three different types of media: one containing rifampicin, one containing chloramphenicol and a one containing both antibiotics. After incubation, students interpret the results and they should find that antibiotic resistance has been transferred from one strain of *E. coli* to the other.



◀ Bacterial conjugation involves the one-way transfer of DNA from a donor ('male') to a recipient ('female') strain, through a tube called a sex pilus. The pilus is made by the donor cell using genes encoded by the plasmid. The DNA passes through the pilus not as a whole, double-stranded plasmid, but as a single strand of DNA which is replicated on arrival in the recipient cell.

Planning the lessons

This investigation takes place over three lessons. The first introduces the concept of horizontal gene flow between bacteria by conjugation and some basic aseptic techniques. Students 'mate' two cultures of bacteria and incubate the donor, recipient and 'mated' strains overnight. In the second lesson, students plate out the bacteria on selective (antibiotic-containing) growth media and learn more advanced aseptic techniques. This also introduces the concept of 'controls' in scientific investigations. In the third lesson, students examine and interpret the results. Here there is an opportunity to discuss the emergence of antibiotic resistance in natural bacterial populations, the healthcare and social consequences of such resistance, and the ways in which microbial evolution differs from that of complex multicellular organisms.

Careful planning and preparation of the growth media and cultures is essential for success in this practical work; there are many materials to prepare and good technical support and understanding of basic microbiological techniques is vital.

Equipment and materials required

Equipment

- An autoclave or pressure cooker
- An incubator, maintained at 30°C
- One or more thermostatically-controlled water baths, set at 50°C
- 500 mL flasks, 3 (for preparing growth media)
- A gas lighter or matches (for lighting the Bunsen burners)
- Access to a hand wash basin with soap and paper towels
- OPTIONAL: Adhesive tape for securing the lids to the Petri dishes

Note: For safety reasons, 30°C is the preferred incubation temperature for E. coli in schools.

Materials

- Slope culture of *Escherichia coli* HT-99
- Slope culture of *Escherichia coli* J53-R
- Chloramphenicol powder, 25 mg
- Rifampicin powder, 100 mg
- Nutrient agar powder, 28 g
- Nutrient broth powder, 13 g
- Petri dishes, sterile, 40
- Universal bottles, sterile, 40
- 1 mL syringes, sterile, 20
- 5 mL syringe, sterile, 1
- 150 mL medical flats, each containing 95 mL of sterile distilled water, 2
- 5 mL pastettes, sterile (individually packed), 4
- Inoculation loops, sterile plastic (individually packed), 54
- A suitable liquid disinfectant, *e.g.* VirKon®
- Methanol, 40 mL
- Aluminium foil
- Distilled or demineralised water
- Autoclavable disposal bags (at least four, for contaminated paper towels, cultures, plates *etc.*)

For each working group

- A Bunsen burner
- A waste container for holding the disinfectant solution, such as a plastic measuring cylinder
- A waterproof marker pen for labelling the plates
- Paper towels for swabbing the benches

Note: Heat-resistant mats should not be used under the Bunsens, as these can make it difficult to clean up any spills effectively.



Protective clothing

Students, teachers and technicians undertaking this work must wear laboratory coats. Protective glasses should be worn if appropriate *e.g.*, when diluting disinfectants.



Disposable gloves should not generally be worn, however, as these are unnecessary and can be hazardous if they catch fire or interfere with manipulation (an exception might be if the person involved has cuts or scratches on their hands which cannot be adequately covered by adhesive plasters). Please refer also to Appendix 1, which gives details of good microbiological practice.



Technician's notes

Preparation of cultures for Lesson 1

For successful conjugation, the bacteria *must* be in an active stage of growth. If cultures are taken straight from agar slopes they are highly unlikely to be at the correct stage of growth. Therefore bacteria from slopes should first be streaked onto nutrient agar plates (without any antibiotics) and incubated at 30 °C for 24 hours. Individual colonies should then be picked off the plates and used to inoculate the nutrient broth cultures that are needed by the students.

The broth cultures should be incubated overnight, starting *the day before* the cultures are needed by the students. **You will therefore need to start preparing the cultures for this work at least two days before the first lesson.** Subculturing a streak plate like this also makes it possible to identify contaminants.

A wise precaution is to prepare *two* plates of each culture from the slopes so that if one does not grow well, or becomes contaminated, you will have a second in reserve.



Slope cultures of the two *E. coli* strains...

...are subcultured onto nutrient agar plates and grown overnight.

Broth cultures are inoculated from the plates and grown overnight for students.

Each working group of students (we suggest that they work in groups of three) will need about 10 mL of each of the two bacterial cultures in nutrient broth (in fact, the students will need less than 10 mL of each culture, but smaller volumes are hard to autoclave and dispense).

If you have ten working groups (that is, 30 students) it will therefore be necessary to prepare 20 x 10 mL bottles of sterile nutrient broth and to inoculate half of them with *E. coli* J-53R and the rest with *E. coli* HT-99. The inoculated broth should then be incubated overnight at 30 °C.

Note that each group of students will also need 10 mL of uninoculated sterile nutrient broth — this can be prepared at the same time as the two cultures.

Lesson 1: Preparing and 'mating' the cultures

The first lesson introduces the concept of horizontal gene flow between bacteria by conjugation and some basic aseptic techniques. Students 'mate' two cultures of bacteria and incubate the donor, recipient and 'mated' strains overnight at 30 °C. Students can be asked to prepare for this lesson by researching bacterial cell structure and mechanisms of microbial gene transfer. The necessity of aseptic techniques should be explained and these techniques must be demonstrated before the students prepare the mixed cultures.

Preparing the cultures should take at most 30 minutes, so the entire lesson should fit comfortably within a 50–60-minute period. This means that there should be time to introduce the topic well and prepare for the following lesson which is more challenging, both intellectually and practically.

Technicians will need to prepare fresh bacterial cultures and liquid media in advance of this lesson. It is essential that these overnight cultures are plated out the following day. It may therefore be necessary to ask students to return in their lunch break to do this.

Equipment and materials

Required by each student or working group

Equipment

- Bunsen burner
- Waterproof marker pen
- Waste container of disinfectant.

Materials

- Overnight culture of *E. coli* J53-R, 10 mL
- Overnight culture of *E. coli* HT-99, 10 mL
- Bottle containing 10 mL of sterile nutrient broth
- Sterile 1 mL syringes (without needles), 2
- Paper towels

Note: A measuring cylinder may be used as a waste container.

Additional equipment required by the class

- Access to an incubator set at 30 °C
- Discard bag for paper towels

*Note: For safety reasons, 30 °C is the preferred incubation temperature for *E. coli* in schools.*



Procedure

1. Wash your hands with soap and water.
2. Wipe down the bench surface with disinfectant. There should be a lit Bunsen burner on the bench near where you are working to create an upward flow of warm air, to carry away potentially contaminating microorganisms.
3. Label the bottle containing sterile nutrient broth: 'Mating'.
4. Open a sterile syringe at the end of the packet *furthest from the tip*, taking care not to touch the barrel of the syringe or the tip. Use the syringe to aseptically remove 1.8 mL of an overnight culture of *E. coli* J53-R and transfer it to the nutrient broth in the bottle you have just labelled. (Since you have a 1 mL syringe, you will need to transfer 2 x 0.9 mL.)

5. Discard the used syringe into the waste container of disinfectant.
6. Taking care as before, use a new sterile syringe to aseptically transfer 0.2 mL of the *E. coli* HT-99 culture to the nutrient broth.
7. Discard the second syringe into the waste container of disinfectant.
8. Place the mixed culture and the other two cultures in an incubator at 30°C (where they should be left for 4–16 hours).
9. Wipe down the bench with disinfectant.
10. Wash your hands with soap and water.

Note: Discarded syringes should be completely immersed in the disinfectant and left for 24 hours before disposal in the normal waste. The disinfectant solution should be discarded after use.



Technician's notes

Preparation of antibiotic plates for Lesson 2

This should be done in advance by the teacher or technician for the whole class. Each student or working group will require three plates:

- one with nutrient agar + rifampicin;
- one with nutrient agar + chloramphenicol; and
- one with nutrient agar + both antibiotics.

The quantities given here are sufficient for 12 sets of plates (36 plates), allowing ~17–20 mL of medium per plate. This number provides two extra plates of each medium in case of accidents, etc.

If you have a smaller class or wish students to work in smaller or larger teams, you will need to adjust the number of plates you prepare accordingly.

Preparing the nutrient agar growth media

1. Prepare three separate batches of nutrient agar by adding 7 g of nutrient agar powder to each of three 500 mL conical flasks. While stirring, to one flask, add 200 mL of distilled or deionised water; two each of the others, add 225 mL of distilled or deionised water.
2. Cover the tops of the flasks with aluminium foil, then autoclave them at 121°C for 15–20 minutes.
3. While the liquid is being autoclaved, the nutrient agar may sink to the bottom of the flasks. Therefore you should swirl the flasks to mix the nutrient agar after autoclaving.
4. Let the flasks cool to 55–60°C (that is, until the flasks can be held comfortably in your hands). While the medium is cooling, label the bases of the sterile Petri dishes: one third with 'NA + R', a third with 'NA + C' and a third with 'NA + RC'.
5. Keep the nutrient agar molten by standing the flasks in a water bath at ~50°C.

Note: if the agar begins to solidify, it can be re-melted by autoclaving again for no more than 5 minutes.



Handling the antibiotics

Wear protective gloves, eye protection and clothing when handling the antibiotics.

Do not allow the antibiotic powder or solutions to come into contact with your skin or eyes. Do not inhale the antibiotic powder.



For additional precautions and first aid instructions, please refer to the MSDS safety sheets that should accompany the antibiotic.

The chloramphenicol and rifampicin powders will need to be dissolved before they are used. The final concentrations required in the growth media are:

- Chloramphenicol: 25 µg per mL
- Rifampicin: 100 µg per mL

Because the antibiotic solutions are heat-labile, it is often more convenient to use powders rather than solutions. Most schools do not have facilities for weighing out very small quantities of these powders however, so you will probably wish to use small pre-weighed amounts to which the solvents can be added. The amounts provided by suppliers will probably make up far more antibiotic solution than is required. *Unused antibiotics should be disposed of with care. Any unused antibiotic solution must be autoclaved to denature it before disposal down the sink with plenty of tap water.*

Although when they are dissolved in methanol and protected from the light the antibiotics can be stored in a fridge for several weeks, once dissolved in water they will deteriorate rapidly.

Preparing the antibiotic solutions

1. Before opening the vials of antibiotic powder, tap each one gently so that the powder falls to the bottom of the vial.
2. Remove the lid of each vial and add an appropriate volume of methanol to each.
3. Replace the lids and agitate the vials until the powders dissolve to give clear solutions. Wrap the vials in aluminium foil to exclude light, which will cause the antibiotics to break down. *The antibiotic solutions may be kept in this form for several weeks in a refrigerator at 3–5 °C until they are needed.*
4. When you are ready to use the antibiotics, transfer the correct volume from each vial to a separate sterile bottle and add 95 mL of sterile water to each. Cap the bottles tightly and agitate each one until clear solutions are obtained. Label the bottles carefully. (Rifampicin solution is bright orange in colour). These antibiotic solutions should be used immediately, but if absolutely necessary they may be stored in a secure fridge at 3–5 °C for up to two days before use.

Preparing the nutrient agar plates

1. About five minutes before you are ready to pour the plates, warm the rifampicin and chloramphenicol solutions by placing the bottles in the same water bath (at 50 °C) as the flasks. *Do*

not leave the antibiotic solutions in the water bath for more than five minutes, as the heat will cause the antibiotics to break down.

2. Wash your hands and wipe down the bench with disinfectant. There should be a lit Bunsen burner on the bench near where you are working to create an upward flow of warm air, to carry away potentially contaminating microorganisms. *Ensure that the flammable antibiotic solutions are not placed near the flame!*
3. Once the antibiotic solutions have warmed, mix them aseptically as described below.

Flask 1, labelled 'NA + R' (containing 225 mL of medium)

Add 25 mL of rifampicin solution to the nutrient agar

Flask 2, labelled 'NA + C' (containing 225 mL of medium)

Add 25 mL of chloramphenicol solution to the nutrient agar

Flask 3, labelled 'NA + RC' (containing 200 mL of medium)

Add 25 mL of rifampicin solution and 25 mL of chloramphenicol solution to the nutrient agar

4. After you have added the antibiotic solutions to the agar, swirl them to mix. ***Important! Do not allow the bottles to cool excessively, as the agar will set. Return the media to the waterbath as necessary to ensure that it remains molten. (If the agar sets, it can only be melted again by boiling it, which will destroy the antibiotics.)***
5. Arrange the Petri dishes on the bench in three groups, ready to pour the medium into them.
6. Lift the lid of each Petri dish in turn, just enough to pour the nutrient agar in. Do not put the lid down on the bench. Quickly add enough agar medium to cover the bottom of the plate to a depth of about 3 mm (17–20 mL). Replace the lid then tilt the plate to spread the medium evenly over the Petri dish.
7. Continue pouring the plates, using the appropriate medium for each. As you do so, flame the mouth of the flask occasionally to maintain sterility.
8. To remove any bubbles from the surface of the poured nutrient agar, *very briefly* touch the surface with a Bunsen burner flame while the medium is still molten.
9. Leave the medium to set, undisturbed, for about 15 minutes.
10. If time permits, invert the plates and incubate them for several hours or overnight at 30°C. This dries the medium, limiting condensation; it also allows any contaminated plates to be detected.
11. Stack the plates in their original plastic sleeves and store them at room temperature.
12. Wipe down the bench with disinfectant.
13. Wash your hands with soap and water.

Although the prepared plates can be kept for up to a week in a refrigerator at 3–5°C, we advise that you do not do this. Plates stored for any length of time in a fridge will accumulate condensation and be difficult to use. It is better to try to make the plates the day before you need them.

Lesson 2: Streaking the plates with the cultures

In the second lesson students plate out the bacteria on selective (antibiotic-containing) growth media and learn more advanced aseptic techniques. These techniques will need to be explained and demonstrated by the teacher (perhaps by now, however, the students will be able to offer explanations for the procedures as they are shown). There are several video recordings showing standard aseptic techniques available on-line (see *Additional resources*), but once again such techniques are best taught practically.

This lesson also covers the concept of 'controls' in scientific investigations. Depending upon the ability of the students, they can be asked in advance to plan the 'controls' that might be required in the investigation. They can also be asked to find information about the two antibiotics involved (rifampicin and chloramphenicol) and the topic of antibiotic resistance in general.

The teacher may need to briefly recapitulate on the process of conjugation and its rôle in the spread of antibiotic resistance and discuss with students what they might expect to see on the various plates after incubation. Again this work should fit comfortably within a 50–60 minute period.

Technicians will need to prepare antibiotic-containing plates for this lesson: these should be incubated for 24 hours at 30 °C, but after that they can be refrigerated and kept for up to a fortnight. This gives greater flexibility in lesson timing should this be required.

An important constraint however, is that the broth cultures (prepared by students in Lesson 1) *must* be prepared no more than 24 hours before this second lesson. If it proves difficult to arrange for students to do this, it could be done for them by a teacher or technician, but ideally students should experience setting this up too.

Equipment and materials Required by each student or working group

Equipment

- Bunsen burner
- Waterproof marker pen
- Waste container of disinfectant

Materials

- Plate of nutrient agar containing rifampicin
- Plate of nutrient agar containing chloramphenicol
- Plate of nutrient agar containing rifampicin and chloramphenicol
- Sterile disposable loops, 3
- The three microbial cultures from the previous lesson

Additional equipment required by the class

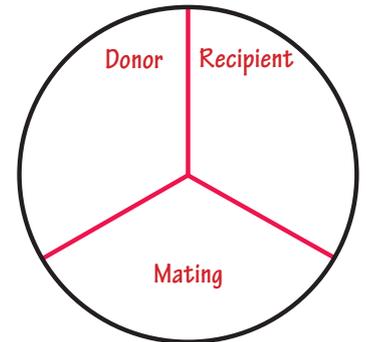
- Access to an incubator set at 30 °C

Note: For safety reasons, 30 °C is the preferred incubation temperature for E. coli in schools.

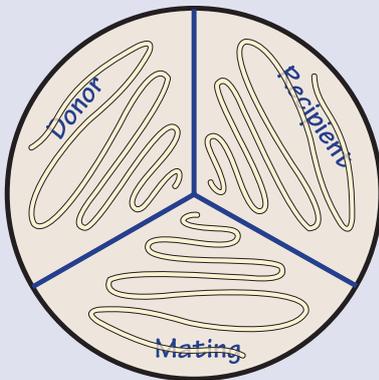


Procedure

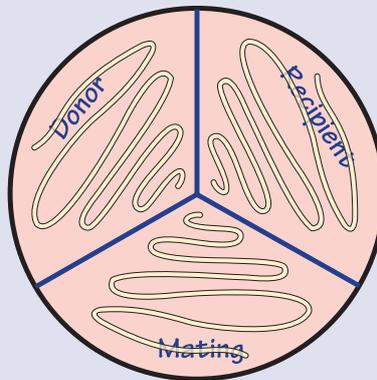
1. Wash your hands with soap and water.
2. Wipe down the bench with disinfectant.
3. Turn over each plate so that the base is uppermost. With the marker pen, divide each plate into three segments as shown in the diagram. Label the top two segments 'Donor' [HT-99] and 'Recipient' [J-53R] and the third segment as 'Mating'.
4. Arrange the three plates on the bench in front of you. Use a new sterile loop to aseptically streak each segment of each of the plates with culture from the appropriate bottle. Use a new loop for each of the different cultures, and dispose of the loops into the disinfectant solution as you use them. The inoculation plan is shown below:



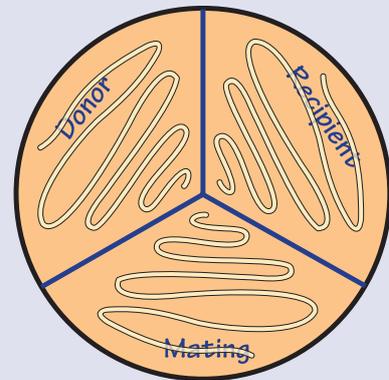
How the base of the plate should be marked



Chloramphenicol plate



Chloramphenicol + Rifampicin plate



Rifampicin plate

5. When the agar has absorbed any excess liquid, invert the plates and incubate them for 24 hours at 30°C.
6. Wipe down the bench with disinfectant.
7. Wash your hands with soap and water.



Lesson 3: Examining and interpreting the results

After incubation, the plates can be stored in a fridge and examined at any time up to a fortnight later. This should make timetabling more convenient for schools. In the third lesson, students examine and interpret the results. This should not take very long. Students may wish to photograph the plates so that they have a permanent record of their work. Here there is an opportunity to discuss the emergence of antibiotic resistance in natural bacterial populations, the health care and social consequences of such resistance, and the ways in which microbial evolution, where genes are passed both 'vertically' and 'horizontally', differs from that of multicellular organisms. It is also a good time to emphasise the fact that all of the cultures will be destroyed after use by autoclaving, to ensure that no contribution is made to the spread of antibiotic resistance.

A suitable worksheet (e.g., with a chart) could be prepared for students to use when interpreting their results.

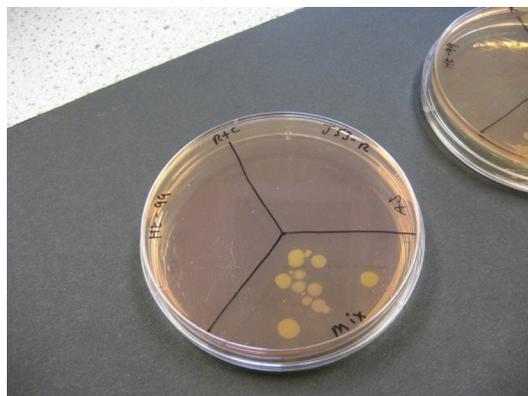
Specimen results



R – Rifampicin plate. Since the donor strain *E. coli* HT-99 is resistant to chloramphenicol and sensitive to rifampicin, it will not grow on this plate.



C – Chloramphenicol plate. The recipient strain *E. coli* J-53R is resistant to rifampicin and sensitive to chloramphenicol, so it will not grow on this plate.



R+C – Rifampicin + chloramphenicol plate. Only the 'mated' *E. coli* can grow, indicating that gene transfer has occurred by conjugation.



Technician's note

Disposal of cultures

It is very important to dispose of all the materials used in a practical class properly, especially cultures of antibiotic-resistant bacteria. All containers used for storing and growing cultures *must* be autoclaved after use to ensure that all microorganisms are killed.

Two autoclave bags should be available in the laboratory: one for reusable glassware and another for disposable materials. There should be a waste discard jar with disinfectant solution near each work area for items such as disposable loops and waste liquid cultures. A bucket should be available in the laboratory or prep room for disposal of any broken glassware.

After use, disinfectant in the waste jars should be poured away and the items in it should be disposed of. Note that contaminated items should be left fully immersed in the disinfectant for *at least 24 hours*. Contaminated paper towels, cloths and plastic Petri dishes should be put into the autoclave bag reserved for disposable items. Any contaminated glassware should be put into the autoclave bag for glassware.

Glassware that is not contaminated can be washed normally. Broken glassware should be put in a waste bin reserved exclusively for that purpose. If the glassware is contaminated it must be autoclaved before disposal. Uncontaminated broken glassware can, of course, be disposed of immediately.

Suppliers

The two strains of *E. coli* required for this practical work are available from the National Centre for Biotechnology Education (NCBE) in the UK. NCBE, University of Reading, 2 Earley Gate, Whiteknights Road, Reading RG6 6BZ United Kingdom. T: + 44 118 9873743 F: + 44 118 9750140 E: NCBE@reading.ac.uk W: www.ncbe.reading.ac.uk

References

1. *Annie's box: Charles Darwin, his daughter and human evolution* by Randal Keynes (2001) London: Fourth Estate. ISBN: 978 1841150604. [This book has been made into a feature film, 'Creation', which will be released in autumn 2009.]
2. Mwangi, M. *et al* (2007) Tracking the *in vitro* evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. *PNAS* **104**: 9451–9456.
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4. Kunin, V. *et al* (2005) The net of life: Reconstructing the microbial phylogenetic network. *Genome Research* **15**: 954–959.
5. *A review of gene transfer from genetically-modified micro-organisms* by T.M. Timms-Wilson, A. K. Lilley and M.J. Bailey (1999) Health and Safety Executive Contract Research Report 221/1999. Norwich: HSE Books. ISBN: 0 1776 2445 5.
6. Rivera, M. and Lake, J. (2004) The ring of life provides evidence for a genome fusion origin of eukaryotes. *Nature* **431**: 152–155.

Other sources of information

The Wellcome Trust

From March 2009, the Wellcome Trust's 'Survival Rivals' web site (www.survivalrivals.org) will include additional resources such as an animated demonstration of bacterial conjugation to accompany this protocol.

Antibiotic resistance

There are numerous web sites with high-quality information on the topic of antibiotic resistance. Here are some examples:

- **PBS (USA) Evolution of antibiotic resistance** (TV programme)
http://www.pbs.org/wgbh/evolution/library/10/4/l_104_03.html
- **UK Department of Health**
<http://www.dh.gov.uk/en/PublicHealth/Patientsafety/Antibioticresistance/index.htm>
- **European Antibiotic Awareness Day**
<http://antibiotic.ecdc.europa.eu/materials.asp>

Microbiology safety (UK resources)

Safety in science education Department for Education and Employment (1996) Her Majesty's Stationery Office. ISBN: 011 270915 X.

Topics in safety Association for Science Education (2001) [Third Edition] Association for Science Education ISBN: 0 86357 316 9.

Basic practical microbiology: A manual by Dariel Burdass (2003) Society for General Microbiology ISBN: 978 0953683833. Available from the SGM: <http://www.sgm.ac.uk>

Microbiology Online

<http://www.microbiologyonline.org.uk>

Manchester Metropolitan University: Good Microbiology Laboratory

Practice videos. Available to download from:

<http://www.sci-eng.mmu.ac.uk/inthelooop>

Acknowledgements

This protocol was originally devised for the Wellcome Trust's 'Survival Rivals' project. 'Survival Rivals' is the Wellcome Trust's *Darwin 200* offering for secondary schools in the UK (www.survivalrivals.org). The Wellcome Trust is an independent charity that funds research to improve human and animal health (Registered charity No 210183).

Thanks also to Leighton Dann and the several UK secondary schools that carried out trials of this practical investigation.

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Appendix 1

Microbiology safety guidelines

Use of *Escherichia coli*

All micro-organisms should be regarded as potentially harmful. However, the strains of the bacterium *E. coli* used in the current investigation present minimum risk given good practice. These laboratory strains should not be confused with the highly-pathogenic *E. coli* O157:H7, other pathogenic strains or the normal 'wild type' *E. coli* that is found in the human gut.

Maintenance and storage of cultures

In schools, microbial cultures are often maintained on agar slopes. These should be transferred onto fresh medium every 8–12 weeks or so, and incubated until the organisms have grown. They should then be stored in a cool (10–15 °C), dark place, not in a refrigerator. New cultures should be obtained regularly, as repeated sub-culturing can lead to contamination or genetic changes in the cells.

Good microbiological practice

General precautions

- Any exposed cuts or abrasions should be protected with waterproof dressings before the practical work starts.
- Everyone involved — teachers, technicians and students — should always wash their hands *before and after* practical work.
- The laboratory door and windows should be closed when work is in progress. This will reduce air movements and consequently the risk of accidental contamination of plates, *etc.*
- Laboratory coats must be worn, and where necessary (*e.g.*, when heating liquids), eye protection.
- High standards of cleanliness must be maintained. Non-porous work surfaces should be used and they should be swabbed with an appropriate laboratory disinfectant before and after each practical session.
- To reduce the risk of ingesting microbes, no hand-to-mouth operations should occur (*e.g.*, chewing pencils, licking labels, mouth pipetting). For the same reason, eating and drinking must not be allowed in the laboratory.

Spills and breakages

Accidents involving cultures should be dealt with as follows:

- Disposable gloves should be worn.
- The broken container and/or spilt culture should be covered with a cloth soaked in disinfectant.
- After not less than 10 minutes, it must be cleared away using paper towels and a dustpan.
- The contaminated material must be placed in an infected waste container or disposal bag. This must be autoclaved before disposal.
- The dustpan should also be autoclaved or placed in a suitable solution of disinfectant for 24 hours.

Contamination of skin or clothing

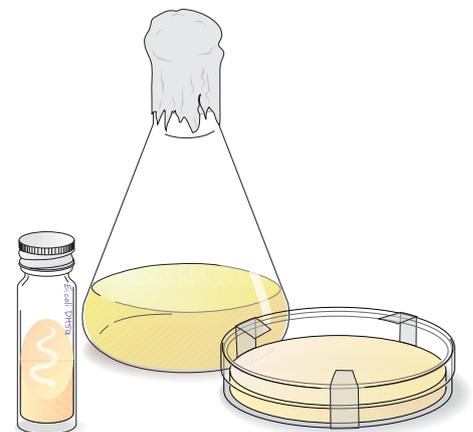
As soon as possible, anyone who has been splashed should wash. Severely contaminated clothing should be placed in disinfectant before it is laundered. Contaminated cleaning cloths should be autoclaved or soaked in disinfectant.

Aseptic techniques

The aims of aseptic techniques are:

- To obtain and maintain pure cultures of microorganisms;
- To make working with microorganisms safer.

A 'pure culture' contains only one species of microorganism, whereas a 'mixed culture' contains two or more species.

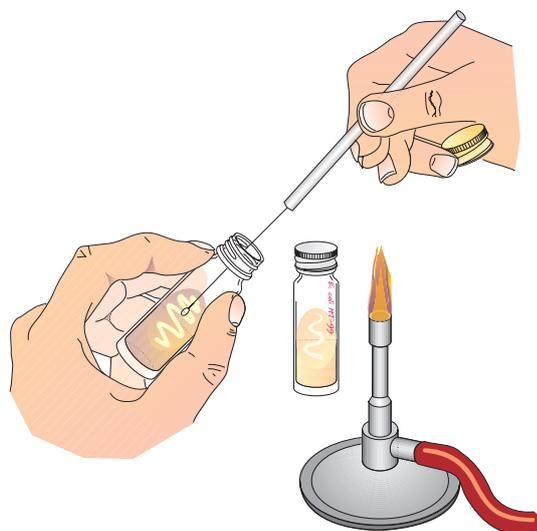


Contamination of cultures is always a threat because microbes are found everywhere; on the skin, in the air and on work surfaces and equipment. To obtain a pure culture, sterile growth media and equipment must therefore be used and contaminants must be excluded. These are the main principles of aseptic techniques.

Growth media must be sterilised before use — usually by autoclaving. Sterile containers (flasks, Petri dishes, *etc.*) should be used. Lids must be kept on these containers to prevent contamination.

It is essential to prepare the work area carefully before you start. The bench surface should be swabbed thoroughly with a suitable disinfectant and allowed to dry. All necessary equipment and materials should be arranged so that they are readily-at-hand. Work should be done near a lit Bunsen burner. Rising air currents from the flame will help to carry away any microbes that could contaminate growth media and pure cultures.

When cultures are transferred between containers, tops and lids should not be removed for any longer than necessary. After a lid has been taken from a bottle, it should be kept in your hand until it is put back on the bottle. This stops contamination of the bench and the culture.



A blue flame about 5 cm high should be used for sterilising wire loops and flaming the necks of bottles. After removal of the top, the neck of the culture bottle should be flamed briefly. This will kill any microbes present there and cause convection currents which will help to prevent accidental contamination of the culture from the atmosphere. Bottles should not be heated until they become hot and dangerous to handle.

Sterile, plastic disposable loops may be used. There is no need to flame these, but they should be opened carefully (at the handle end) immediately

before use, used, then discarded immediately into a suitable disinfectant. A similar procedure should be adopted with the sterile, plastic disposable syringes.

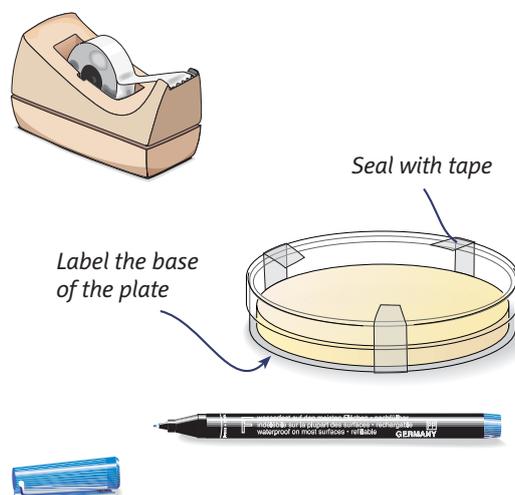
If you use wire loops, however, these must be heated until they glow red hot along the entire length of the wire part. This should be done both before and after cultures are transferred. Heat the stem of the loop first as it is brought into the Bunsen burner flame, to reduce sputtering and aerosol formation. Allow the loop to cool before you use it to transfer a culture (some people like to cool the loop after flaming it, by touching it briefly onto the agar at the edge of a culture plate).

When the Bunsen burner is not in use, it should be kept on a visible yellow flame.

Incubation

Label the Petri dish around the edge of the base before inoculation. Your name, date and the name and/or source of the organism used will allow the plate and its contents to be identified.

Where appropriate, use self-adhesive tape to seal Petri dishes. The seal will ensure that the plates are not accidentally opened or tampered with. Do not seal plates completely round their edges as this could create anaerobic growth conditions within the dish.



Bacterial cultures in Petri dishes should usually be incubated with the base uppermost, so that any condensation that forms falls into the lid and not on the colonies. (If there is heavy condensation in the sterile Petri dish before inoculation, it should be allowed to dry before use.)

The strains of *E. coli* used in this investigation should be incubated at 30 °C — this is the maximum incubation temperature usually recommended for use in schools.

Disposal and sterilisation

It is very important to dispose of all the materials used in a practical class properly. All containers used for storing and growing cultures must be autoclaved, then washed and rinsed as necessary, before re-use.

At least two autoclave bags should be available in the laboratory: one for reusable glassware and another for disposable materials. There should be a discard jar near each work area for materials such as waste liquid cultures and pipettes. A bucket should be available for disposal of any uncontaminated broken glassware.

After use, disinfectant in the discard jar should be poured away and the items in it should be autoclaved and disposed of. Contaminated paper towels, cloths and plastic Petri dishes should be put into the autoclave bag reserved for disposable items. Any contaminated glassware should be put into the autoclave bag for glassware.

Glassware that is not contaminated can be washed normally. Broken glassware should be put in a waste bin reserved exclusively for that purpose. If the glassware is contaminated it must be autoclaved before disposal. Uncontaminated broken glassware can, of course, be disposed of immediately.

Autoclaving

Sterilisation is the complete destruction of all microorganisms, including their spores. All non-sterile equipment and media should be sterilised before starting practical work so that there are no contaminants. Cultures and any contaminated material should also be sterilised after use for safe disposal.

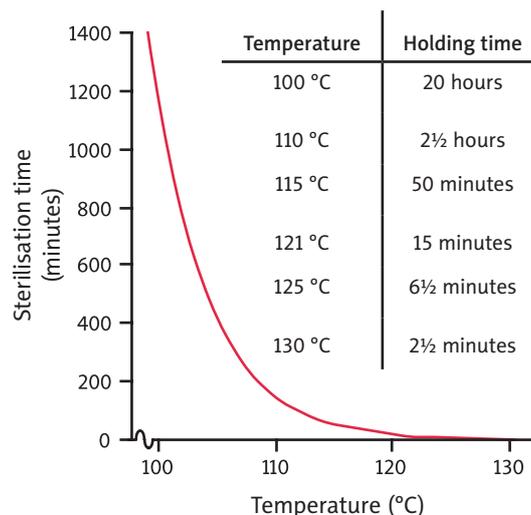
Autoclaving is the preferred method of sterilisation for culture media, aqueous solutions and discarded cultures. The process uses high pressure steam, usually at 121 °C. Microbes are more readily killed by moist heat than dry heat as the steam denatures their proteins. A domestic pressure cooker or a purpose-built autoclave can be used. Domestic pressure cookers can be used in school laboratories but their small capacity can be a disadvantage when dealing with class sets of material.

Principles of autoclaving

Two factors are critical to the effectiveness of the process. Firstly, all air must be driven from the autoclave. This ensures that high temperature steam comes into contact with the surfaces to be sterilised: if air is present the temperature at the same steam pressure is lower. The materials to be sterilised should be packed loosely so that the air can be driven off. Screw-capped bottles and jars must have their lids loosened slightly to allow air

to escape and to prevent a dangerous build-up of pressure inside them.

Secondly, sufficient time must be given for heat to penetrate (by conduction) to the centre of media in flasks or other containers. The times for which media or apparatus must be held at various temperatures for sterilisation are shown below:



Notice that just a small difference in temperature can result in a great difference in the time required for sterilisation. It is also important that these temperatures are reached by all materials to be sterilised for the specified time *e.g.*, the broth in the very centre of a flask. Three factors determine the duration of the autoclaving process:

- penetration time: the time taken for the innermost part of the autoclave's contents to reach the required temperature (say, 5 minutes);
- holding time: the minimum time in which, at a given temperature, all living organisms will be killed (say, 15 minutes);
- safety margin: roughly half the holding time (about 5 minutes).

Most domestic pressure cookers and autoclaves operate at 121 °C. This gives a total autoclaving period of around 25 minutes. For larger volumes of liquid, such as 1 litre of growth medium in a flask, the holding time should be increased to 20–25 minutes.

The effectiveness of an autoclave can be checked by using autoclave test strips which change colour if the process has worked properly (autoclave tape, which also changes colour after heating, does not show this).

Use and routine care of autoclaves

Different autoclaves and pressure cookers will have different operating instructions and it is important that the manufacturer's instructions are always followed. Care should be taken to ensure that there is enough water in the autoclave so that it does not boil dry during operation. A domestic pressure cooker requires at least 250 mL of water — larger autoclaves may need much greater volumes. The use of distilled or deionised water in the autoclave will prevent the build-up of limescale or corrosion of the metal pressure vessel. Autoclaves should be dried carefully before storage to prevent damage to the vessel.

When the autoclave is used, steam should be allowed to flow freely from it for about one minute to drive off all the air inside. Only then should the exit valve be tightened. After the autoclave cycle is complete, sufficient time must be allowed for the contents to cool and return to normal atmospheric pressure. Premature release of the lid and the subsequent reduction in pressure will cause any liquid inside the autoclave to boil. Therefore the vessel or valve must not be opened whilst under pressure as this may cause scalding and the agar or broth will froth up and may boil over the outside of the containers within.

**Chemical sterilisation**

WARNING! Eye protection should be worn when dispensing concentrated disinfectant solutions.

Many different chemicals are used for sterilisation of used equipment and work surfaces. Some disinfectants, such as *VirKon*[®] can be safely used for most laboratory purposes and are recommended for use in schools. Others have specialist uses. The manufacturer's and supplier's instructions should always be followed with care.

Disposable plastic items

Modern microbiology makes extensive use of disposable plastic items, such as Petri dishes, single-use pipettes and loops. Although they can appear wasteful, we strongly recommend their use as they are inexpensive, save on preparation time and enhance safety by reducing the risk of contamination. After use, such items can be sterilised and disposed of in the normal waste.