BM327 microbiology S1 lab manual

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Table of contents

# Preface

## Introduction to the BM327 Microbiology Semester 1 Lab Manual

This laboratory manual contains key information about each of the five practical laboratory sessions delivered as part of BM327 microbiology semester 1.

### How to use this manual

You can navigate through this manual using the sidebar on the left, or by flipping through the pages (forward/reverse arrows at the bottom of each page.

The introductory section is a general section containing helpful information on good laboratory technique, working with microorganisms safely and aseptically, etc. - it also contains general information about the module, including the assessment. You should read through this section before the first lab and refer back to it when needed to refresh your memory.

Each of the experiment sections 1-5 contain a brief explanation of the theory underpinning the experiments we will do in the practical labs, the protocols that will be used for that experiment, and an explanation of any data analysis tasks that need to be performed after the lab sessions. Links to the protocols (handouts) for each experiment are provided, but please note that you do not need to print these out - they will be provided for you in the laboratories.

### How to prepare for each lab session

You should read through the relevant theory/introductory material before coming to each practical lab session, and make sure you understand it. In some instances you may wish to review the material covered in BM330 before the lab as well (or review the underpinning microbiology theory in an introductory microbiology textbook.

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| Tips for success |
| * You will also find callout boxes (such as this one) throughout this lab manual with Tips, Important notes, Discussion Questions, To Do Lists, etc. These are intended to help you prepare for each lab (and for the assessment), so you should make sure to read through all the material. * As Benjamin Franklin said, “By failing to prepare, you are preparing to fail.” It is really important that you come prepared to each lab! A little bit of time spent reading through the material and performing any necessary tasks or calculations will really pay off. |

### How to deal with any problems, questions, or concerns

If you have any problems or questions, please feel free to speak with Dr. Feeney during the lab sessions or by e-mail.

If you are going to miss a lab session (for whatever reason), you should e-mail Dr. Feeney and also log your absence with the university using the Pegasus Personal Circumstances tool. More details can be found in your student handbook.

If you notice any errors (including typos, broken links, etc.) in this lab manual, please notify Dr Feeney, either by e-mail or by [raising an issue](https://github.com/mafeeney/BM327microS1/issues/new) on this lab manual’s github page.

# 1. Aim of the BM327 Semester 1 Microbiology laboratory sessions

The aim of this block of laboratory sessions is to introduce students to fundamental molecular biology and microbiology methods, such as UV mutagenesis, plasmid transfer, antibiotic assays and microscopy.

Within the block of five three-hour laboratory sessions, there are five distinct experimental elements. Each experimental element is linked to the others as part of an overall research project. Most of the experimental elements will take place over the course of several different laboratory sections (because of the need to incubate plates and allow microbes to grow). There are also three workshops (“CAL labs”) to supplement your learning – details of these will be made available on MyPlace.

## 1.1 Microbiology Laboratories Competency Skills

Graduate recruiters look for specific job skills and competencies. Competencies are action-oriented statements of the knowledge, skills, and abilities people need to do their work successfully. They are observable and measurable. These are what you need to do the job and work in their organisation. Some are straightforward and required in many different career: communication, teamwork and problem solving are just a few. You need to show employers you have mastered essential skills such as writing, reading, numeracy, presentation, organisation and the ability to work under pressure. Graduates who demonstrate their analytical thinking during a job interview or assessment centre will stand out from other candidates. Other skills such as those gained in the laboratory are more specific and aligned to particular types of jobs e.g. ability to do a PCR.

You will be assessed for your competency in the following:

*Lab 1*

* Good laboratory practice (labelling, pipetting, aseptic technique)
* Performing basic lab calculations, e.g. serial dilutions
* Performing data analysis
* Interpreting phenotypic and genotypic data
* Teamwork
* Keeping detailed and accurate records

*Lab 2*

* Good laboratory practice (aseptic technique, pipetting, spreading plates)
* Experimentally determining antibiotic MBC and MIC values
* Teamwork
* Keeping detailed and accurate records
* Performing data analysis

*Lab 3*

* Good laboratory practice (aseptic technique, pipetting, labelling, spreading plates)
* Performing data analysis (mapping loci on a bacterial chromosome)
* Using online databases
* Teamwork
* Keeping detailed and accurate records

*Lab 4*

* Performing basic lab calculations
* Good laboratory practice (pipetting, labelling, handling DNA, gel electrophoresis)
* Performing data analysis
* Formulating hypotheses
* Teamwork
* Keeping detailed and accurate records

*Lab 5*

* Good laboratory practice (microscopy)
* Data analysis
* Data presentation
* Teamwork
* Keeping detailed and accurate records

## 1.2 Overall learning outcomes

* To understand how exposure to UV radiation can affect the ability of microorganisms to survive and produce antibiotics.
* To understand how genetic elements are transferred between bacteria.
* To understand how the susceptibility of bacteria to certain antibiotics is determined.
* To observe how bacterial cells can change their morphology as a result of certain environmental stimuli.

# 2. Schedule of experimental tasks

Because of the nature of microbiology (cultures require incubation and take time to grow), many of your experiments will take place across more than one laboratory session, as detailed in the following schedule ([Figure 2.1](#fig-schedule)). It is therefore essential that you keep careful track of all experimental tasks: e.g., carefully labelling your cultures and taking care to read all the relevant sections of the lab manual.

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| Figure 2.1: Schedule of experimental tasks |

# 3. Preparing for each lab session

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| To prepare for each lab |
| Before attending each of the practical labs, you should ensure that you have read and understood:   * The introductory sections covering [Good Lab Practice](./0goodlab.qmd) and [Aseptic Technique](./0aseptic.qmd); and (where relevant), those covering [Enumerating Microbes](./0counting.qmd) and [Microscopy](./0microscope.qmd) * The introductory sections covering the theory underpinning each experiment * The protocols we will be using in that lab (you do not need to print these out - we will provide you with a handout) * You also must ensure that you have read and signed the safety forms, or you will not be admitted to the lab. |

# 4. Formative assessment

## 4.1 Summative assessment: Laboratory Report

The assessment for the BM327 semester 1 microbiology block of laboratories will be based on the labs and the workshops. The pro forma that you should complete for this assessment is available on MyPlace. It is due on 24/11/2025, at noon, and should be submitted via MyPlace.

### 4.1.1 Summative assessment: General Guidelines

1. **Correct Formatting of Figure Titles and Legends** Each figure must be accompanied by a clear and concise figure title and legend.
2. **Correct Formatting of gene and species names.**

The correct binomial name (genus and species) should be given for each microbe. In the first instance, the species name must always be given in full (e.g. *Escherichia coli*); subsequently, it may be abbreviated (e.g. *E. coli*) provided that there can be no confusion with other genera mentioned in the work. Species names must always be italicized.

Gene and protein names should be correctly formatted. In bacteria, gene names are written in italics, with the first letter in lower-case (e.g. *yfgA*); protein names are not italicized, and the first letter is in upper-case (e.g. YfgA).

If in doubt, refer to the [Journal of Bacteriology nomenclature guidelines](https://jb.asm.org/content/nomenclature)

1. Correct Formatting of References: You should reference relevant papers where necessary.

References should be listed in a reference list/ section using a standard formats. Articles published in peer-reviewed journals are referenced in the standard format: Authors’ surname and initials, (year of publication), article title, abbreviated journal title, volume, pages.

Simply including the link to the web page you downloaded the paper from is NOT acceptable.

Example: Lederberg, J. and E. Tatum. (1946). Gene recombination in *E. coli*. Nature, 158: 558.

### 4.1.2 Contribution to overall class assessment

The BM327 semester 1 microbiology assessment comprises 25% of the overall BM327 class assessment.

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| Important |
| IMPORTANT NOTE: A link to all Safety Paperwork (eCOSHH and eRisk Assessments) for each appropriate laboratory will be sent to you in an email a few days before the session. These electronic documents MUST be read ahead of each laboratory and acknowledgement of this indicated by ticking the box that is at the end of the document. This MUST be done at least ONE day ahead of the session. Failure to do this will result in you not being allowed into the laboratory session. A link to the electronic document will also be available on the appropriate BM327 Being a Biomolecular Scientist 3 class sub-page on Myplace.  It is essential that you prepare thoroughly for these labs by reviewing each section and that you understand fully what you are doing in each lab. If in doubt, ask a member of staff. |

# 6. Good Laboratory Practice

# 7. Good Lab Practice

Many of the principles for working in a microbiology lab are the same as those used in any other biomedical research lab. These are transferable skills, which are useful for a number of different careers.

Good lab practice is a cornerstone of biomedical research: it helps to ensure that results are reproducible, and is especially crucial for any clinical or therapeutic work.

## 7.1 Keeping a Laboratory Notebook

It is essential to keep an accurate record of experiments, with details of every experiment performed, the data collected, and the data analysis that was performed. It is just as important to keep a lab notebook for experiments performed in silico as it is for experiments performed at the lab bench.

You should be able to come back to your notebook six months later, read your notes, and be able to recreate the experiment/understand the analyses that you performed. Human memory is fallible and it is important to keep clear, accurate records.

Moreover, an appropriately kept lab notebook is a legal document, important for protecting your intellectual property. You should therefore be in the habit of keeping your lab notebook up to date and accurate.

### 7.1.1 Some guidelines for keeping a good lab notebook:

* Your notebook may be kept in the form of a physical notebook (kept on paper) or an electronic notebook. The former should be written in clearly and legibly, using indelible ink. The latter should be saved often, with backup copies stored securely.
* Include the date for each entry.
* Each experiment should include a title, a hypothesis or aim, a brief description of the background, protocols and details of any reagents/equipment used, observations, and any data or statistical analysis that was performed.
* Any protocols or methods should be described in enough detail that a colleague (or you in six months’ time!) can repeat the same experiment, starting from the same dataset/materials, and reach the same results.
* Any mistakes should be crossed out and explained (not erased).
* All data should always be recorded in your lab notebook (even “failed” experiments or “outliers”).

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| Electronic Lab Notebooks |
| Increasingly, researchers are using electronic lab notebooks instead of paper ones - these are more impervious to physical destruction (e.g., chemical spills, lab fires) and often more convenient (more easily legible, searchable, etc.) However, care must be taken to ensure that the data stored in an electronic lab notebook is secure and backed up.  There are many options for lab notebooks. One popular and widely used electronic lab notebook is Benchling.   * You can join Benchling by creating an account at http://benchling.com/signup * For help with Benchling, including training kits and best practice guides, please see https://help.benchling.com/hc/en-us. |

### 7.1.2 Good Labelling Technique

It is essential that all tubes and plates be clearly and legibly labelled. You will often be working with these over a period of several weeks, and you will be unable to interpret/analyse the results from a previous week’s experiments if you have not labelled your samples correctly.

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| Figure 7.1: Label around the rim of the Petri plate - not across the centre |

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| Figure 7.2: Label the bottom of the Petri plate, not the lid |

**Some general guidelines for good labeling practice:**

* Include your initials and the date of the experiment (if directed by your instructors, also include your bench number).
* If working with microorganisms, always include the species name (abbreviations, e.g. *E. coli* are fine).
* If labeling Petri plates, label around the outside rim of the plate (do not obscure the plate as it will make it hard to count colonies!), as shown in [Figure 7.1](#fig-platelabelling). You should also be sure to label the *bottom* of the plate ([Figure 7.2](#fig-platelid)). The bottom and lid may become separated, so labeling the lid is a bad idea!

### 7.1.3 Other tips for good lab practice

* Read through each protocol thoroughly before beginning an experiment. Label all tubes and prepare any reagents needed before you begin. Stay organised and keep your space clean.
* Turn off your Bunsen burner (or set it to a visible orange flame) if you are not working aseptically.
* Never walk away from your bench and leave a lit Bunsen burner unattended.
* Never wear gloves when working with a Bunsen burner.
* Pipet as accurately as possible:
  + Use the correct pipet for the volume you intend to pipet and make sure it is set correctly (if uncertain, ask a demonstrator or member of staff)
  + Pipet down to the first stop when drawing up liquid into the pipet; pipet down to the second stop when expelling liquid.
  + Avoid bubbles when pipetting.
  + Only insert the very tip of the pipet into the liquid when pipetting particularly viscous mixtures (e.g., polymerase or enzyme stocks).

# 8. Aseptic Technique

## 8.1 Aseptic Technique

It is **essential** that you work aseptically when working in a microbiology lab. Microbes are ubiquitous in the environment (including in the air, and on your skin). Therefore, you must work aseptically so that your experiment contains only the organism(s) you are working with, and not unwanted contaminants!

Most of the materials you will be working with (pipet tips, microcentrifuge (Eppendorf) tubes, culture media, etc. will have been sterilised for you by autoclaving: the autoclave sterilises these items by subjecting them to pressurised steam (121°C, 15 p.s.i.).

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| Autoclaved items |
| You will be able to recognise that items such as tips or Eppendorf tubes have been autoclaved based on the use of autoclave tape on the containers for these items. Autoclave tape changes colour when subjected to high heat and pressure. It is essential that you use these sterilised items: do not take unsterilised pipet tips or microcentrifuge tubes from the shelves.   |  | | --- | | Figure 8.1: The use of autoclave tape to show that the contents of a container have been sterilised (compared to unautoclaved tape, on the right). Image credit: Wikipedia | |

One of the cornerstone principles of aseptic technique is that any objects, even those that have previously been sterile, can no longer be considered sterile if they come into contact with non-sterile objects. Be careful not to allow your pipet tips to come into contact with any non-sterile surfaces (i.e., your bench top, fingers, the lids or outsides of bottles).

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| Top Tip for Good Aseptic Technique |
| You should use good aseptic technique when working with all containers (including tip boxes, Eppendorf boxes, bottles of media, etc.), to prevent the contents from becoming contaminated.  Containers should be opened for the minimum amount of time possible (the longer they are open, the greater the chance of contamination!) Close them promptly when you have removed the tip/sample.  Tip boxes should **not** be left open. If you leave the box open, microbes present in the air can easily contaminate the tips.   |  | | --- | | Figure 8.2: A box of pipet tips that are no longer sterile. Image credit: Starlab |   You should therefore always: 1. Open tip box –> 2. Take pipet tip –> 3. Close tip box –> 4. Use pipet tip |

### 8.1.1 Working with a Bunsen Burner

In order to keep your work sterile and free from contamination that could interfere with your experiments, you should work near a Bunsen burner set to a blue flame ([Figure 8.3](#fig-bunsen)). The Bunsen creates an updraft of air, helping to maintain a “clean” zone around it. Having the Bunsen burner lit, but working several meters away from this clean zone, is ineffective and will likely result in contamination of your experiments.

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| Figure 8.3: A Bunsen burner set to a blue flame. Image credit: Encyclopedia Britannica |

When working with a Bunsen burner, however, it is essential that you follow the following safety guidelines, for the safety of yourself and those working near you:

* Turn off your Bunsen burner (or set it to a visible orange flame) if you are not actively using it.
* Never walk away from your bench and leave a lit Bunsen burner unattended.
* Never wear gloves when working with a Bunsen burner.

#### 8.1.1.1 Using a platinum inoculating loop

In some cases you will need to use a platinum inoculating loop, e.g. to streak bacteria for single colonies on a new Petri plate.

You must make sure to sterilise your inoculating loop by flaming it (placing the platinum wire in the flame of your Bunsen burner to sterilise it). You should flame your loop both before touching it to a sample (this prevents the transfer of contaminants into your sample) and before putting it down when you have finished using it (so that you do not transfer microbes to the lab bench/other surfaces.

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| Important |
| An inoculating loop that has just been flamed is extremely hot - you should never allow a hot loop to touch your skin. You should also avoid touching a hot loop directly to your samples, as the heat will kill the microbes you are working with. |

The procedure for flaming an inoculating loop is:

1. Place the loop in the flame of the Bunsen burner at an angle, so that the length of the loop lies along the surface between the inner and outer cones in the flame (the hottest part of the flame).
2. Hold the loop in the flame until the entirety of the loop turns cherry-red. It is not necessary to hold the loop there any longer than that: you are not trying to melt the metal!
3. Remove the loop from the flame and allow it to cool, either by holding it in the air for several seconds (in the aseptic working zone near the Bunsen burner), or by touching it to a sterile surface (i.e. the inside of a culture tube or the agar in an uninoculated Petri dish.)

### 8.1.2 Top tips for working with good aseptic technique:

* Work **near** a Bunsen burner set to a blue flame (Many students end up working miles away from their Bunsen, which is not very helpful.)
* Open all containers (including tip boxes, Eppendorf boxes, bottles of media, etc.) for the minimum amount of time possible (the longer they are open, the greater the chance of contamination!) Close them promptly when you have removed the tip/sample. Tip boxes should not be left open.
* Be careful not to allow your pipet tips to come into contact with any non-sterile surfaces (i.e., your bench top, fingers, the lids or outsides of bottles).

# 9. Using a Microscope

## 9.1 Correct use of a microscope

Bacteria are much smaller than anything else you will have looked at under a microscope; most bacterial cells are approximately 1-3 µm long. Optimal set up of the microscope is therefore essential.

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| Safety tip for working with microscopes |
| Please note that you should not wear gloves while working with a microscope: you do not want to inadvertently transfer staining reagents (or other chemicals) onto it. |

You should begin by placing a slide onto the stage and aligning the lowest power objective (usually the 10X objective) over it.

1. Raise the stage using the coarse focusing knob so that the slide is about 5 mm away from the objective.
2. Bring the slide into focus as best you can, first using the coarse focusing knob and then the fine focusing knob. You will be slowly lowering the stage so as to increase the distance between the slide and objective.
3. Once you have focussed as best you can using this objective, you should move on to the next highest power objective.

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| Finding the focal plane |
| Often the plane of focus is extremely narrow, so that small, faint objects like stained bacteria may be easily missed as the focus passes through the plane.  Thus, a good technique is to constantly move the focus up and down while also slowly moving the slide up/down or left/right on the stage. In this way, objects that flash in and out of focus tend to be more easily noticed by the eye. |

At any point while using the microscope, you may wish to:

* Adjust the “interocular separation” of the eyepieces so that they are comfortably aligned with your eyes.
* Adjust the lamp voltage at any time when using a microscope in order to get a comfortable level of light.

### 9.1.1 Use of oil with the microscope:

* The 100x objective is designed to be used with oil (it will have “oil” explicitly written on it), whereas the other objectives are not.
* Never use oil with any objective other than the 100x. Once you have added oil to your sample, never return to using a smaller magnification objective!
* Only a very small amount of oil is needed, only add a tiny drop. Don’t flood the slide!
* When you have found your focus and optimised set-up with the 40x objective, swing the objectives half-way between the 40x and 100x. DO NOT move the stage or change the focus as you do this! Then gently add a very small drop of oil onto the spot of light you can see in your sample. Gently swing the 100x objective into place.
* You will most likely have to adjust the focus a little, but only use the fine adjustment at this stage. Ask for help if you have trouble finding the focus. Under no circumstances go back to the 40x, as it does not tolerate getting in contact to the immersion oil.
* Residual oil will cling to the 100X objective after use, so make sure you clean it off with a lens tissue (specifically designed for this purpose) before putting the objective away. DO NOT use any other tissue to clean the lens.

# 10. Serial Dilutions

It is often necessary or desirable to work backwards from the number of colonies on a plate, in order to work out the number of *viable* microbes that were present in the original (undiluted) sample. (Note that any microbes present in the sample that were dead, or viable-but-not-culturable, will not give rise to colonies on the plate - so you are enumerating the number of viable microbes, not the number of cells.)

We calculate and express the number of viable microbes in the original sample in **colony forming units** (cfu) - usually in cfu/mL.

For example, if you performed a decimal serial dilution, plated 0.1 mL of the 10-4 dilution, and counted 173 colonies on the resulting plate ([Figure 10.1](#fig-serialdilplated)) - how many viable bacterial cells were present in the original culture?

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| Figure 10.1: Examples of plating and counting colonies from a decimal serial dilution |

Here, we had 173 colonies in 0.1 mL of the dilution present in the 10-4 dilution tube.

To calculate the number of cfu/mL present in the original sample, divide the number of colonies on your plate by the total dilution factor: in this case, 173 colonies/(0.1 mL \* 10-4) = 1.73 \* 107 cfu/mL.

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| Top tips for calculating cfu/mL |
| * Make sure you label your plates with the dilution factor - you will need this for the calculations! * Ideally, you want to count colonies on a plate that has 30-300 colonies. We normally consider <30 colonies to be Too Few To Count (TFTC) and more than 300 colonies to be Too Numerous To Count (TNTC). You will get more accurate estimates of your viable cell count if you use a countable plate. * Make sure you use the correct number of significant figures in your answer - you can’t be more precise than your initial measurement (number of colonies counted). * Make sure you express your answer in scientific notation (e.g., 1.73 \* 107 cfu/mL, not 17300000) and include units. * When you’ve finished your calculation, double-check that your answer makes sense! For example, you can’t have a fraction of a colony forming unit - so if your answer looks like 1.73 \* 10-7 cfu/mL, you have probably gone awry with your handling of exponents somewhere. |

# 11. Experiment 1 Aims and Overview

## 11.1 Aims

* To understand how exposure to mutagens can affect the ability of microorganisms to survive and produce antibiotics.

## 11.2 Learning Outcomes

At the end of this experiment, you should understand:

* How mutagenesis can affect the phenotype of an organism.
* That caffeine is a potent mutagen.
* That mutagens act in a dose-dependent manner.
* How to analyse a mutagenesis data set.

## 11.3 Competencies

* Good laboratory practice (labelling, pipetting, aseptic technique)
* Performing basic lab calculations, e.g. serial dilutions
* Performing data analysis
* Interpreting phenotypic and genotypic data
* Teamwork
* Keeping detailed and accurate records

## 11.4 Importance

This practical session aligns with lectures in BM330 Fundamental Microbiology and reinforces material relating to: bacterial genetics, mutagenesis, establishing kill kinetics in sterilisation processes, the induction of DNA damage by mutagens in industrial microbiology and strain improvement.

Mutagens like caffeine and UV damage DNA and, at high enough doses, are lethal. Caffeine and UV have long been used as potent, effective mutagens by bacterial geneticists: UV causes the formation of pyrimidine-pyrimidine dimers, and inhibits excision-repair mechanisms for DNA. If left unrepaired pyrimidine-pyrimidine dimers lead to DNA replication errors (mutations). Caffeine and UV are relatively non-site specific mutagens, and can be used to introduce mutations randomly throughout a genome. These mutations may cause different phenotypic changes in the bacteria being studied, depending on the location and nature of the mutation.

## 11.5 Overview of Experiment 1

You will perform Protocol 1.1 in the Week 2 lab and collect your data the following session (Week 4). Perform the data analysis tasks ([Chapter 18](#sec-data1)) using your own data/the class data or the sample data available on MyPlace.

# 12. Experiment 1 - Background

## 12.1 Experiment 1 - Background

We have isolated a mutant strain of Streptomyces coelicolor A3(2) that produces a novel antibiotic that can kill Escherichia coli and other Gram-negative bacteria. However, the Streptomyces strain only produces small amounts of the antibiotic.

In order to study this antibiotic further, you will carry out a UV mutagenesis experiment, and screen for mutants that produce more antibiotic ([Chapter 16](#sec-protocols1)).

Your task is to determine the most effective dose of UV radiation to use for isolating mutants ([Section 18.1](#sec-1Adata)). You will then analyse the phenotypes of mutants isolated at this dose ([Section 18.1.1](#sec-1Bdata)).

We have selected a number of candidate genes that might be involved in regulating antibiotic production, and sequenced these genes in a set of mutant strains. Your final task for this experiment is to examine the potentially mutant allele sequences, and identify any amino acid change(s) compared to the wild-type sequence (Workshop 1).

# 13. Streptomyces and Antibiotic Production

## 13.1 Streptomyces - Introduction

The growing antimicrobial resistance (AMR) crisis is estimated to cause or contribute to a large number of deaths annually (e.g., 1.27 million deaths in 2019 as a direct consequence of bacterial AMR[[1]](#footnote-112)).

One part of the scientific efforts to tackle this growing crisis includes the search for novel antibiotics.

Many of the antibiotics in current use are produced by bacteria: in particular, by actinobacteria, including *Streptomyces*.

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| Streptomyces |
| Streptomyces are Gram-positive, filamentous, actinobacteria that are commonly found in the soil and many other environments.  These bacteria typically have large, linear chromosomes with a high GC content (usually >70%). They also have a unique, complex developmental life cycle. However, they are probably best known for their prolific production of antibiotics and other pharmaceutically useful compounds (e.g., anti-cancer drugs, immunosuppressants). |

Most strains of *Streptomyces* that have been identified or sequenced contain relatively large number of biosynthetic gene clusters (BGCs) in their genome (for example, *Streptomyces coelicolor* A3(2) has >20 such BGCs).

While some of these BGCs have been linked to specific molecules (e.g., for *S. coelicolor* A3(2), antibiotics such as the (usually) blue-pigmented actinorhodin and the red-pigmented undecylprodigiosin, and other small molecules such as siderophores or signalling molecules) - many of these BGCs are not expressed under known growth conditions, and the molecules produced remain elusive.

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| Antibiotic Discovery |
| What approaches do you think could be used to discover new antibiotics from *Streptomyces* or other bacteria? What experiments would you do? |

# 14. Bacterial Mutagenesis

## 14.1 Bacterial Mutagenesis - Introduction

Mutagenesis is the process by which the genetic material of an organism is changed, resulting in changes (mutations) from the original (“wild-type”) genetic sequence. In bacteria, mutagenesis is widely used as a tool to generate genetic diversity, study gene function, and create strains with desirable traits (e.g., improved growth characteristics in bioreactors/industrial fermentations, resistance to environmental stresses, or enhanced production of compounds such as antibiotics).

Researchers often apply mutagenic treatments to large populations of bacterial cells, then **screen** or **select** for mutants showing altered phenotypes. Two of the most commonly used methods are **UV mutagenesis** and **chemical mutagenesis**, which produce random, untargeted mutations throughout the genome.

**UV mutagenesis** exposes bacterial cultures to ultraviolet (UV) light, typically UV-C (around 254 nm), which induces the formation of pyrimidine dimers in DNA. These lesions can lead to errors during DNA replication, generating mutations.

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| UV mutagenesis - dose |
| Because UV light is lethal at high doses, it is important to experimentally determine the correct dose of UV that should be administered to a particular bacterial species or strain during a UV mutagenesis experiment. If the dose is too high, all of the bacteria in the sample will be killed; however, if it is too low, there will be a large number of unmutated bacteria still present, and it becomes tedious to screen through all of these for the desired phenotype. Therefore, UV treatments are usually calibrated by determining a “kill curve” that leaves only a small fraction of surviving, potentially mutated cells. |

**Chemical mutagenesis** uses chemicals that interact with DNA to cause base substitutions, insertions, or deletions. Common agents include alkylating compounds such as ethyl methanesulfonate (EMS) or nitrosoguanidine (NTG), which modify DNA bases and increase replication errors. Chemical mutagens can produce a high density of random mutations throughout the genome, creating a broad pool of genetic variants. However, each particular chemical mutagen will have a specific signature (the type of mutation that it causes, based on its chemical structure and mode of action - for example, EMS alkylates guanine residues, resulting in G:C to A:T **transition** mutations.)

**Transposon (Tn) mutagenesis**, by contrast, introduces mutations by inserting a mobile genetic element (transposon) into random sites in the genome. Because the sequence of the transposon is known, the exact location of the mutation can be easily identified afterwards using techniques such as inverse PCR or sequencing across the transposon–genome junction. This makes Tn mutagenesis especially powerful because it can be used to rapidly link genotype to phenotype (whereas UV and chemical mutagenesis often require additional mapping steps to locate the relevant mutation).

Random mutagenesis (UV, chemical, or Tn-based) can reveal new genetic variants that would not arise under natural mutation rates or that would not be discovered using targeted (site-directed) mutagenesis approaches, making it a powerful approach for basic research and applied biotechnology.

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| Bacterial Mutagenesis |
| * Why does the UV dose (“kill curve”) need to be determined experimentally for each particular bacterial species or strain? * When carrying out a bacterial mutagenesis experiment, would you prefer to screen or select for a phenotype of interest? Why? * How can we determine if there is a causal link between a particular mutation and a phenotype of interest? |

# 15. Experiment 1 Protocols

# 16. Experiment 1 - Protocols

You can view the protocols for experiment 1 below, or download it [here](./assets/Experiment1protocols.pdf).

# 17. Experiment 1 - Data Analysis

# 18. Experiment 1 - Data Analysis

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| Experiment 1 - Data |
| The data you should have from this lab are:   * Colony counts from the caffeine mutagenesis experiment (your data/class data on MyPlace) * Colony counts from the UV mutagenesis data (experiment performed for you and data posted on MyPlace).   The UV data shows you three plates for each time point, starting at T=0 (un-irradiated spores) and then the number of colonies present on the plates after the time indicated, and the dilution of the spores to obtain that count. The times are T = 0 (un-irradiated spores); 30 seconds (0.5 min) UV treatment; 1 min UV treatment; 2 min UV treatment; 4 min UV treatment; 8 min UV treatment and 16 min UV treatment. Each plate contains 1 ml of spores. |

## 18.1 Task 1A - Plot “kill curves” for UV and/or caffeine

1. You should make the following calculations:
2. Using the triplicate counts for each time point, work out the mean CFU survival following UV irradiation or caffeine treatment of the spores for each time point.
3. Using these data plot a UV dose versus survival graph of your data (x-axis = time; y-axis = CFU/ml).
4. Using the data, work out the percentage survival of spores following irradiation or caffeine treatment. Establish the time-point that gives >99% spore killing. (x-axis = time; y-axis = percentage spore survival). Un-irradiated spores will be 100%

### 18.1.1 Task 1B - Analyse mutant phenotypes

1. Compare the colonies obtained on the UV-irradiated plates with those on the equivalent dilutions of unirradiated plates (data available on MyPlace).
2. Carefully record your data (observations) in an appropriate format.
3. Can you identify over-producing mutants of the pigmented antibiotic Actinorhodin (Blue) or undecylprodigiosin (Red)? Do the colonies look different after UV mutagenesis? after caffeine mutagenesis?
4. We have already selected a number of mutants for further analysis – but which mutants would you have selected (and why)?

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| Experiment 1 - discussion questions |
| * Compare the two mutagens we used (UV and caffeine). Which is more effective in terms of killing ([Section 18.1](#sec-1Adata))? in terms of generating mutants with the desired phenotypes ([Section 18.1.1](#sec-1Bdata))? * Which colonies would you select for further analysis? * What experiments would you like to do to characterize these mutant strains? * What are the advantages and disadvantages of using UV mutagenesis? Caffeine mutagenesis? |

# 19. Additional Resources/Suggested Further Reading for Experiment 1

## 19.1 Streptomyces

* Bentley SD, Chater KF, Cerdeño-Tárraga AM, et al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature. 2002;417(6885):141-147. doi:10.1038/417141a
* Hoskisson PA, van Wezel GP. *Streptomyces coelicolor*. Trends Microbiol. 2019;27(5):468-469. doi:10.1016/j.tim.2018.12.008
* Challis GL. Exploitation of the *Streptomyces coelicolor* A3(2) genome sequence for discovery of new natural products and biosynthetic pathways. J Ind Microbiol Biotechnol. 2014;41(2):219-232. doi:10.1007/s10295-013-1383-2
* Schlimpert S, Elliot MA. The Best of Both Worlds-*Streptomyces coelicolor* and *Streptomyces venezuelae* as Model Species for Studying Antibiotic Production and Bacterial Multicellular Development. J Bacteriol. 2023;205(7):e0015323. doi:10.1128/jb.00153-23

## 19.2 Bacterial mutagenesis

* Nolan LM, Webber MA, Filloux A. Throwing a spotlight on genomic dark matter: The power and potential of transposon-insertion sequencing. J Biol Chem. 2025;301(6):110231. doi:10.1016/j.jbc.2025.110231
* Banaś AK, Zgłobicki P, Kowalska E, Bażant A, Dziga D, Strzałka W. All You Need Is Light. Photorepair of UV-Induced Pyrimidine Dimers. Genes (Basel). 2020;11(11):1304. Published 2020 Nov 4. doi:10.3390/genes11111304
* Cronan JE. Approaches to Study Proteins Encoded by Essential Genes. Proteins. Published online August 15, 2025. doi:10.1002/prot.70039
* Shuman HA, Silhavy TJ. The art and design of genetic screens: *Escherichia coli*. Nat Rev Genet. 2003;4(6):419-431. doi:10.1038/nrg1087
* Craney A, Ahmed S, Nodwell J. Towards a new science of secondary metabolism. J Antibiot (Tokyo). 2013;66(7):387-400. doi:10.1038/ja.2013.25
* Bennett JA, Kandell GV, Kirk SG, McCormick JR. Visual and Microscopic Evaluation of *Streptomyces* Developmental Mutants. J Vis Exp. 2018;(139):57373. Published 2018 Sep 12. doi:10.3791/57373
* [Evelyn Witkin (Rutgers): The SOS Response in Bacteria](https://youtu.be/NFSkSlDTxxs?si=OmTz1MZacFobeha8) (iBiology Science Stories)
* [Biology Simulations: Mutation](https://www.biologysimulations.com/mutation) (simulation of a bacterial mutagenesis lab)

# 20. Experiment 2 Aims and Overview

# 21. Experiment 2: Determination of minimum inhibitory concentration (MIC), minimum bacteriocidal concentration (MBC) and bacterial susceptibility to antibiotics

## 21.1 Aims

* To understand how the susceptibility of bacteria to certain antibiotics is determined.

## 21.2 Learning Outcomes

At the end of this laboratory session, you should understand:

* How the susceptibility of bacteria to certain antibiotics is determined.
* How to design an experiment to determine the MIC of an antibacterial agent.
* How to be able to calculate MIC and MBC.

## 21.3 Competencies

* Good laboratory practice (aseptic technique, pipetting, spreading plates)
* Experimentally determining antibiotic MBC and MIC values
* Teamwork
* Keeping detailed and accurate records
* Performing data analysis

## 21.4 Importance

This practical aligns with the BM330 lecture “Killing Microorganisms” by Dr. Arnaud Javelle and with lectures in BM329 Biomedical Microbiology, and reinforces material relating to: biomedical microbiology, antibiotic use, antibiotic resistance, clinical assessment of antibiotic resistance, mechanisms of resistance, use of antimicrobials.

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a micro-organism after overnight incubation. MICs are used by diagnostic laboratories, mainly to confirm resistance, but most often as a research tool to determine the in-vitro activity of new antimicrobials. Minimum bacteriocidal concentration (MBC) indicates the lowest concentration of antimicrobial that causes cell death and is often different to MIC. The Kirby-Bauer Disk-Diffusion Method is used to assess the susceptibility of a bacterial strain to a variety of antibiotics. A lawn of the test organism is spread on an agar plate and 3-4 antibiotic-containing paper discs are placed on it. After incubation, zones of inhibition appear around each zone and the diameter of each zone indicates if the test organism is resistant or susceptible to a specific antibiotic.

## 21.5 Overview of Experiment 2

You will perform Protocols 2.1 and 2.4 in the Week 4 lab and collect your data in the following sessions (Weeks 6 & 8). Perform the data analysis tasks ([Chapter 26](#sec-data2)) using your own data or the sample data available on MyPlace.

# 22. Experiment 2 - Background

## 22.1 Experiment 1 - Background

We have isolated a strain of *Streptomyces coelicolor* A3(2) that produces a novel antibiotic “X” that can kill *Escherichia coli* and other Gram-negative bacteria. In the previous lab, you isolated mutant strains that may produce higher levels of antibiotic.

In order to determine whether these strains do, in fact, produce increased amounts of antibiotic, you will examine bioassay data for the wild-type and mutant strains ([Section 26.1](#sec-task2A)). Then, you will determine the MIC and MBC for the purified antibiotic against *E. coli* and compare the efficiency of the antibiotic “X” with other known antibiotics ([Section 26.2](#sec-task2B)).

Finally, you are interested in determining the mechanism of action of this novel antibiotic. To do so, you want to isolate a mutant strain of *E. coli* that is resistant to the antibiotic “X”. Your final task is to measure the MIC and MBC of the antibiotic for these mutant strain and compare to the data obtained for your wild-type strain ([Section 26.3](#sec-task2C)).

# 23. Antibiotic Susceptibility

## 23.1 Antibiotic Susceptibility - Introduction

Antibiotics are compounds that inhibit or kill bacteria, and they act through a range of molecular mechanisms. Major classes target essential cellular processes, including cell wall synthesis, protein synthesis, nucleic acid synthesis, and metabolic pathways. However, the effectiveness of any given antibiotic can vary markedly between bacterial species due to differences in cell envelope structure (e.g. the outer membrane barrier in Gram-negative bacteria), efflux pump systems, target site variants, and the presence or absence of specific resistance genes. Even within a single species, strains may differ in susceptibility due to acquisition of plasmid-borne resistance genes or chromosomal mutations.

Several experimental approaches are used to quantify bacterial susceptibility.

* The **minimum inhibitory concentration (MIC)** is the lowest concentration of an antibiotic that prevents visible growth after incubation, typically determined using liquid (broth) cultures containing different antibiotic concentrations.
* The **minimum bactericidal concentration (MBC)** goes further to determine the lowest concentration capable of killing the bacteria. After MIC testing, aliquots from non-growing cultures are plated onto antibiotic-free media, and the lowest concentration that kills ≥99.9% of the initial inoculum is recorded as the MBC. While MIC measures growth inhibition, MBC assesses bactericidal activity, so the two values can differ significantly for bacteriostatic versus bactericidal drugs.
* The **Kirby–Bauer disk diffusion assay** is a qualitative or semi-quantitative method in which antibiotic-impregnated disks are placed on agar lawns, and the diameter of the growth inhibition zone is measured after incubation. Although disk diffusion does not yield exact concentrations, standardized interpretive breakpoints allow clinicians to categorize isolates as susceptible, intermediate, or resistant.

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| Antibiotic Susceptibility - caveats |
| Experimental conditions can strongly affect the outcome of these assays. Media composition, pH, aeration, and inoculum density can influence antibiotic activity and bacterial physiology, so strict adherence to standardized protocols is essential. Some antibiotics are inactivated by specific media components, while growth conditions (e.g., stationary phase growth) can affect bacterial susceptibility by global physiological and gene expression changes. |

While classical phenotypic assays remain the gold standard, new approaches such as whole-genome sequencing, transcriptomics, and proteomics are increasingly being developed to identify resistance determinants and attempt to predict resistance phenotypes.

# 24. Experiment 2 Protocols

You can view the protocols for experiment 2 below, or download it [here](./assets/Experiment2protocols.pdf).

# 25. Experiment 2 - Data Analysis

# 26. Experiment 2 - Data Analysis

You have 2 sets of data to analyse for Experiment 2:

* *Streptomyces* bioassay data (available for you on MyPlace) - you will use these data in [Section 26.1](#sec-task2A).
* Your own MIC and MBC data for Antibiotic X (or the class data available on MyPlace) - you will use these data in [Section 26.2](#sec-task2B).
* MIC/MBC data for antibiotic X against mutant *E. coli* strains - you will use these data in [Section 26.3](#sec-task2C).

## 26.1 TASK 2A. Analyse *Streptomyces* bioassay data

1. You will be provided with a set of data (pictures of bioassays) posted on MyPlace.
2. Note the zone of inhibition around the *Streptomyces* and carefully record your data (observations).
3. What conclusions can you draw about the mutant strains?
4. Which further experiments might you perform to prove that the mutated strain produces more antibiotic “X” than its parent wild-type strain?

## 26.2 TASK 2B. Determine the MIC and MBC for the purified antibiotic

1. You should have a set of your own data, but there are backup data available on MyPlace in case you need them.

* For the MIC data, determine the lowest concentration of antibiotic that inhibits growth (measured by turbidity in the culture tube). This corresponds to the MIC of the antibiotic X for this strain of *E. coli*. You will need to calculate the final concentration of “X” in each tube!
* For the MBC data, determine the lowest concentration of antibiotic that causes cell death (measured by plates where no growth is observable). This corresponds to the MBC of the antibiotic X for this strain of *E. coli*.
* For each Kirby-Bauer disk diffusion plate, measure the diameter of the zone of growth inhibition around each disk to the nearest whole mm.

## 26.3 TASK 2C. Determine the MIC and MBC for the purified antibiotic against mutant *E. coli* strains

1. We have used transposon mutagenesis to isolate some mutant *E. coli* strains that may be more resistant to Antibiotic X. You will be provided with a set of data posted on MyPlace.
2. Perform the same steps as outlined in Task 2B, for this set of data.

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| Experiment 2 - Discussion questions |
| * We used transposon mutagenesis to isolate some mutant strains of *E. coli* that may be more resistant to Antibiotic X. How does this method work? * Are there other approaches we could have used (apart from transposon mutagenesis)? What are their advantages/disadvantages? * What conclusions can you draw based upon the experiments we performed? * What further experiments might you do (and why?) |

# 27. Additional Resources/Further Reading for Experiment 2

* [Mueller-Hinton Medium (Small Things Considered blog post)](https://schaechter.asmblog.org/schaechter/2020/06/mueller-hinton-medium.html)
* [Video demonstration - determining MIC and MBC for hydrogen peroxide](https://www.youtube.com/watch?v=jCShFlXPcmg)
* [Video demonstration - Kirby Bauer disk diffusion method](https://www.youtube.com/watch?v=BXr_kcki4Ag)
* [The evolution of bacteria on a “mega-plate” Petri dish (Kishony lab)](https://www.youtube.com/watch?v=plVk4NVIUh8)
* van Belkum A, Burnham CD, Rossen JWA, Mallard F, Rochas O, Dunne WM Jr. Innovative and rapid antimicrobial susceptibility testing systems. Nat Rev Microbiol. 2020;18(5):299-311. doi:10.1038/s41579-020-0327-x
* [The dearth of antibiotic development - Editors in Conversation (American Society for Microbiology)](https://www.youtube.com/watch?v=XlRd1YSx8e8)

# 28. Experiment 3 Aims and Overview

## 28.1 Aims

* To understand how genetic elements are transferred between bacteria.

## 28.2 Learning Outcomes

At the end of this laboratory session, you should understand:

* That horizontal gene transfer (HGT) can occur between two strains of bacteria
* That HGT can affect the phenotype of an organism
* That plasmid transfer occurs in a specific order
* How to analyse the results of a conjugative transfer experiment

## 28.3 Competencies

* Good laboratory practice (aseptic technique, pipetting, labelling, spreading plates)
* Performing data analysis (mapping loci on a bacterial chromosome)
* Using online databases
* Teamwork
* Keeping detailed and accurate records

## 28.4 Importance

This practical session aligns with lectures in BM330 Fundamental Microbiology and reinforces material relating to: horizontal gene transfer, bacterial genetics.

## 28.5 Isolation of a new antibiotic from *Streptomyces coelicolor*: Mapping mutations that confer antibiotic resistance

You have isolated a strain of *Streptomyces coelicolor* A3(2) that produces a novel antibiotic that can kill *Escherichia coli* and other Gram-negative bacteria. In the previous lab, you isolated *E. coli* strains that are resistant to this particular antibiotic.

In order to identify the mutation(s) that give rise to the antibiotic resistance phenotype, you will physically map the location of the mutations using interrupted mating. You will first analyse the data from the interrupted mating experiment ([Section 32.1](#sec-task3A)), and then form a hypothesis about the location of the antibiotic-resistance mutation ([Section 32.2](#sec-task3B)).

## 28.6 Overview of Experiment 3

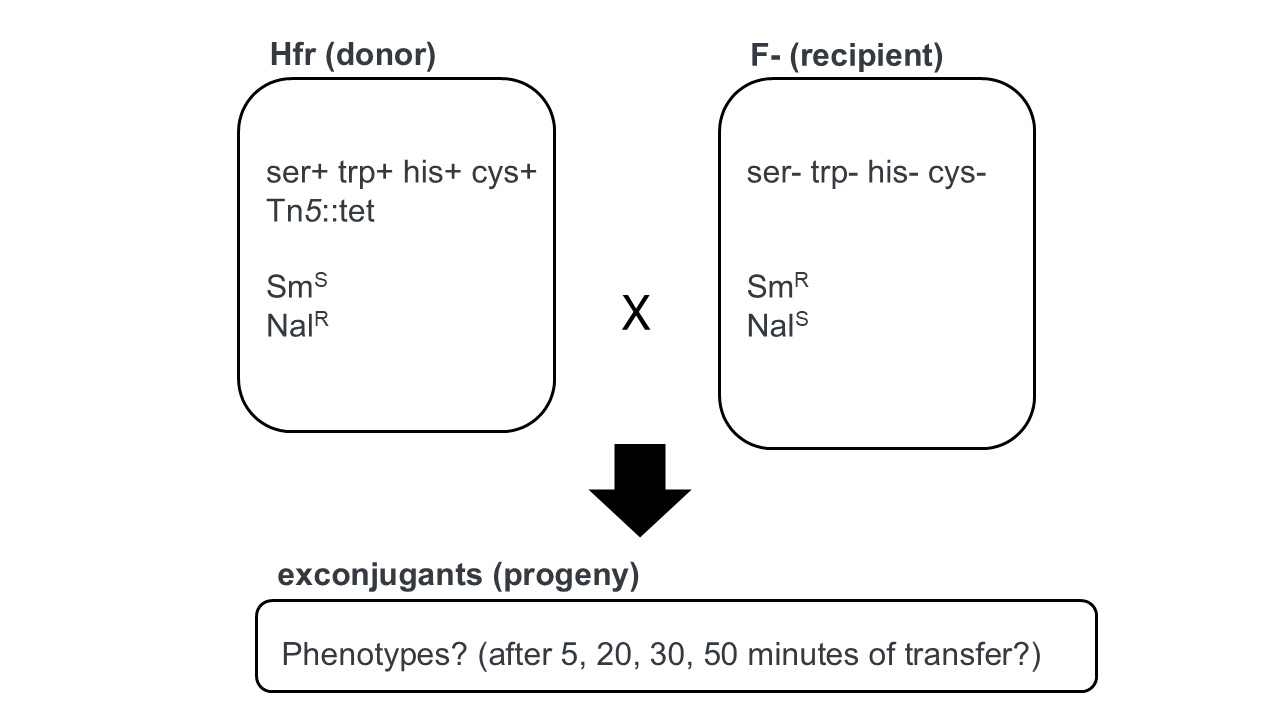
You will perform Protocol 3.1 in the Week 6 lab and collect your data the following session (Week 8). Perform the data analysis tasks ([Chapter 32](#sec-data3)) using your own data/the class data or the sample data available on MyPlace.

# 29. Experiment 3 - Intro

In this practical we will be using the unidirectional exchange of genes between strains of the gut bacterium *Escherichia coli*, in order to map the location of the mutations isolated in the previous lab. You will be provided with an Hfr donor strain which is streptomycin sensitive and nalidixic acid resistant and which carries the sex factor F integrated into the bacterial chromosome. The Hfr donor strain carries the Tn*5* insertion that confers resistance to your novel antibiotic.

The Hfr strain is prototrophic and grows on minimal media in the absence of the antibiotic streptomycin.

The second strain provided is a recipient strain F- ser- trp- his- cys-, naladixic acid sensitive and streptomycin resistant. The F- strain is auxotrophic and will only grow in the presence of supplements of serine, tryptophan, histidine and cysteine. The F- strain is sensitive to your novel antibiotic.

 When the Hfr and F- strains are mixed together, mating occurs and conjugation tubes are formed between strains (**?@fig-HfrCross**). The presence of the F factor in the Hfr strain mobilises the bacterial chromosome and it is transferred down the conjugation tube into the F- strain. Thus gene transfer occurs in a unidirectional manner. In this practical we will be measuring the transfer of genes for serine, tryptophan, histidine and cysteine requirements, as well as the transfer of the Tn*5*s. Each individual Hfr strain transfers its genes in a specific orientation and we will be determining this order for the five genes.

If the mating mixtures are allowed to remain undisturbed a copy of the Hfr chromosome will eventually be transferred to the F- strain. However, if the mating figures are broken apart by physical agitation then only those genes transferred up to the time of agitation will be transferred to the F- strain. Thus, whether or not a gene has been transferred to the F- provides a measure of the order of transfer by the Hfr. If mating figures are broken at specific times, the frequency of each gene transferred provides a measure of the time of transfer. This is called an “interrupted mating experiment.”

# 30. Experiment 3 Protocols

You can view the protocols for experiment 3 below, or download it [here](./assets/Experiment3protocols.pdf).

# 31. Experiment 3 - Data Analysis

# 32. Experiment 3 - Data Analysis

Data you will use for this [Section 32.1](#sec-task3A) and [Section 32.2](#sec-task3B):

* Plate counts from the interrupted mating experiment you performed in week 6 (your data; the plates you counted in week 8)
* Plate counts from a similar interrupted mating experiment that included the Tn mutant strains (available for you on MyPlace)

## 32.1 TASK 3A. Data analysis (interrupted mating)

1. You have colony counts from your own experiment (for the 4 auxotrophic markers) and have been provided with a set of data for the Tn insertions (available on MyPlace). Count the colonies on your plates/the images of the plates and record the data. Fill a table with the number of colonies that you have on each plate.

## 32.2 TASK 3B. Mapping your mutation to the *E. coli* chromosome

1. Using the data from [Section 32.1](#sec-task3A), draw a map of the *E. coli* chromosome, taking into account that it is circular and 100 minutes long.
2. Map the location of each auxotrophic mutation as well as the tetracycline resistant mutation (Tn5).
3. Analyse the region of the chromosome you have identified using [EcoCyc](https://ecocyc.org/) or a similar database. Where on the chromosome do you think your mutation is? (answer in # of minutes) What gene(s) are present at that location?

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| Experiment 3 Discussion Questions |
| * We used interrupted mating to map the location of the Tn*5* insertions in your mutant *E. coli* strains (these Tn insertions are presumably responsible for making the strains resistant to your novel antibiotic). How well did this technique do at mapping the location of the transposons? * Are there any other techniques you could use to identify the location of the Tn*5* insertions? * What conclusions can you draw based upon the experiments performed in this lab? * What further experiments might you do (and why?) * How does your experiment relate to other observations of antibiotic resistance genes being transferred between bacteria by horizontal gene transfer? |

# 33. Additional Resources/Further Reading for Experiment 3

* [Bacterial Gene Mapping using Conjugation](https://www.youtube.com/watch?v=5jV0KdnHiJM) - a very helpful demonstration using string
* [Genetic Recombination and Gene Mapping](https://www.youtube.com/watch?v=TU44tR0hJ8A) - example using *Drosophila*, but still a helpful explanation
* Berlyn MK. Linkage map of *Escherichia coli* K-12, edition 10: the traditional map. Microbiol Mol Biol Rev. 1998;62(3):814-984. doi:10.1128/MMBR.62.3.814-984.1998

# 34. Experiment 4 Aims and Overview

## 34.1 Aims

* To understand how mutagenesis can be used in the laboratory to understand the phenotype of a microorganism

## 34.2 Learning Outcomes

At the end of this laboratory session, you should understand:

* How transposon mutagenesis can affect the phenotype of an organism.
* How to identify the insertion site of a transposon.
* How to predict the effects of a transposon insertion.
* How to correlate physical mapping data (interrupted mating) and sequencing data (plasmid rescue)

## 34.3 Competencies

* Performing basic lab calculations
* Good laboratory practice (pipetting, labelling, handling DNA, gel electrophoresis)
* Performing data analysis
* Formulating hypotheses
* Teamwork
* Keeping detailed and accurate records

## 34.4 Importance

This practical session aligns with lectures in BM330 Fundamental Microbiology and reinforces material relating to: horizontal gene transfer, bacterial genetics.

We have isolated a species of *Streptomyces* that produces a novel antibiotic that can kill *Escherichia coli* and other Gram-negative bacteria. We have also isolated *E. coli* strains that are resistant to this particular antibiotic and, in the previous labs, you identified the location of these antibiotic resistance mutations on the chromosome using interrupted mating.

In order to identify the mutation(s) that give rise to the antibiotic resistance phenotype, you will use a plasmid rescue experiment to identify the insertion site of the Tn*5* transposon in your mutant strain. You will map the plasmid by using restriction digest (Protocol 4.1 and Task 4A).

To “rescue” the Tn*5* from the chromosomes of your mutant strains, we digested chromosomal DNA with the restriction enzyme AflIII, then ligated this into a similarly-digested vector plasmid. We will now check the resulting plasmid by restriction digest: plasmids with insert should produce a band upon AflIII digestion; and we also know that the enzyme HindIII cuts within the Tn*5* sequence.

Once we have verified that the isolated plasmids contain the “rescued” Tn*5*s, you will then analyse DNA sequences derived from the plasmid (Task 4B) to identify the insertion site and generate a hypothesis as to why your mutant strain is resistant to the antibiotic (Task 4C).

## 34.5 Overview of Experiment 4

You will perform Protocol 4.1 and 4.2 in the Week 8 lab and your data will be posted to MyPlace for you. Perform the data analysis tasks (Tasks 4A-C) using your agarose gel data/sequence data posted on MyPlace.

# 35. Experiment 4 - Background

## 35.1 Experiment 4 - Background

A fundamental difficulty in biology lies in our ability to link genotype and phenotype: for example, as in this experiment where we will try to link the known phenotype (antibiotic resistance) with the unknown mutation causing the phenotype (location of the Tn*5* insertion).

In this lab we will be performing a “plasmid rescue” experiment to “rescue” the Tn*5* inserted into the chromosome of the mutant strains. This technique uses restriction enzymes that randomly cut chromosomal DNA isolated from the mutant bacteria; this is then ligated into a vector, and *E. coli* DH5α cells are transformed with this ligation mixture. Transformed cells which carry the region of the chromosome carrying the Tn*5* insertion, will be resistant to tetracycline. The plasmid containing this DNA is isolated from the transformants and Sanger sequencing used to identify the exact position of the Tn5 insertion.

Restriction enzymes are invaluable tools in molecular microbiology. These enzymes make site-specific cuts in DNA (at positions termed restriction sites), and are a key part of the innate immune defences that bacteria use to protect themselves against incoming foreign DNA. Restriction enzymes have found a multitude of applications in molecular biology labs, including their use in cloning, digestion of DNA for Southern blotting, identification of nucleotide differences using restriction fragment length polymorphism (RFLP) analysis, etc. Being able to carry out a restriction digest of DNA, and analyse the results, is therefore a key skill for any microbiologist or molecular biologist.

# 36. Experiment 4 Protocols

You can view the protocols for experiment 4 below, or download it [here](./assets/Experiment4protocols.pdf).

# 37. Experiment 4 - Data Analysis

# 38. Experiment 4 - Data Analysis

You have 2 sets of data to analyse for Experiment 4:

* The images of the agarose gels you ran in the lab (available for you on MyPlace) - You will use these data in [Section 38.1](#sec-task4A).
* Sequence data from these plasmids (available for you on MyPlace) - You will use these data in workshop 3.

## 38.1 TASK 4A. Plasmid rescue

1. The data from the lab (your agarose gel pictures) will be made available for you on MyPlace. Download the image of your gel, and label the lanes correctly.
2. Use the DNA ladder to estimate the size of the bands from your restriction digests (either by eye, or by using the DNA ladder to generate a standard curve of DNA size vs. distance migrated).
3. Draw a map of the plasmid, including all known restriction sites and any other pertinent information (such as the location of the Tn*5*), keeping in mind that the plasmid is circular.

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| Top tips for restriction mapping of a plasmid |
| * Think logically - e.g., if a circular piece of DNA is cut once, how many bands will it produce? What if it is cut twice? * Keep the limits of agarose gel electrophoresis in mind - e.g., it may be difficult to precisely estimate band sizes in some cases, and/or you might have two or more bands that don’t resolve well (depending on your experiment and electrophoresis conditions) * Check out a [worked example to see how someone else performs this task](https://www.youtube.com/watch?v=uNvF9ZBufTk) |

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| Experiment 4 Discussion Questions |
| * We used plasmid rescue followed by sequencing to identify the location of the Tn*5* insertions in your mutant *E. coli* strains (these Tn insertions are presumably responsible for making the strains resistant to your novel antibiotic). How well did this technique do at mapping the location of the transposons? How effective was it compared to the mapping experiment you performed (Experiment 3)? * Are there any other techniques or methods you could use to identify the Tn location (instead of plasmid rescue and sequencing)? * What conclusions can you draw based upon the experiments performed in this lab? * What further experiments might you do (and why?) |

# 39. Additional Resources/Further Reading for Experiment 4

* [Introduction to transposable elements (Susan Wessler lecture)](https://www.youtube.com/watch?v=uNvF9ZBufTk)
* Cain AK, Barquist L, Goodman AL, Paulsen IT, Parkhill J, van Opijnen T. A decade of advances in transposon-insertion sequencing. Nat Rev Genet. 2020;21(9):526-540. doi:10.1038/s41576-020-0244-x
* Warner IA, Kok WJ, Martinelli N, Yang Z, Goodall ECA, Henderson I. Microbial Primer: Transposon directed insertion site sequencing (TraDIS): A high throughput method for linking genotype to phenotype. Microbiology (Reading). 2023;169(11):001385. doi:10.1099/mic.0.001385
* Roberts RJ. How restriction enzymes became the workhorses of molecular biology. Proc Natl Acad Sci U S A. 2005;102(17):5905-5908. doi:10.1073/pnas.0500923102
* [REBASE The Restriction Enzyme Database](https://rebase.neb.com/rebase/rebase.html)

# 40. Experiment 5 Aims and Overview

## 40.1 Aims

* To demonstrate the ability of some microorganisms to alter their cellular morphology in response to environmental stimuli.

## 40.2 Learning Outcomes

At the end of this laboratory session, you should understand:

* How to visualise cells using a light microscope
* How to use ImageJ to process and analyse microscopy data.
* The process of *E. coli* filamentation.
* The process of *Candida* filamentation.

## 40.3 Competencies

* Good laboratory practice (microscopy)
* Data analysis
* Data presentation
* Teamwork
* Keeping detailed and accurate records

## 40.4 Importance

This practical session aligns with lectures in BM330 Fundamental Microbiology and reinforces material relating to: bacterial morphology, killing bacteria, microbial growth kinetics.

You have isolated a species of *Streptomyces* that produces a novel antibiotic that can kill *Escherichia coli* and other Gram-negative bacteria. In the previous labs, you isolated resistant *E. coli* strains and generated a hypothesis about the mechanism of action of the antibiotic.

In order to further study the antibiotic mechanism of action, you will use microscopy to determine whether antibiotic X has any effect on an unrelated species (*Candida albicans*). You will set up cultures with varying concentrations of Antibiotic X and examine the phenotype of antibiotic-treated cells (Protocols 5.1 and 5.2). For comparison’s sake, we have provided you with micrographs of antibiotic X-treated *E. coli* cells ([Chapter 44](#sec-data5)). You will look at a time course of the response of wild-type *E. coli* to the antibiotic, and also, will analyse the phenotype of your mutant *E. coli* strain treated with the antibiotic.

## 40.5 Overview of Experiment 5

You will set up the cultures needed for this experiment in Week 8, and perform the experiment (protocol 5.2) in the following session (Week 10). You will compare/contrast the data you collect in this experiment with the data collected in a similar experiment performed on *E. coli* (posted on MyPlace for you) – Tasks 5A-C).

# 41. Experiment 5 - Background

Cell size and shape are key phenotypes in bacteria and fungi, important for survival in a variety of environments. Bacteria actively modify and adapt their cell size/shape in response to environmental cues including nutrient availability, to adapt to different environmental niches, or as part of developmental pathways. For fungi, the transition between yeast and hyphal growth is often associated with virulence. In order to monitor and understand these changes in cell size and shape, microbiologists use a variety of techniques, including basic light microscopy – using the same principles that allowed the first visualization of microbial cells.

# 42. Experiment 5 Protocols

You can view the protocols for experiment 5 below, or download it [here](./assets/Experiment5protocols.pdf).

# 43. Experiment 5 Data Analysis

# 44. Experiment 5 - Data Analysis

In addition to the *Candida* experiment you performed in the lab, we have also grown *E. coli* cells (wild-type and antibiotic X-resistant) in the presence and absence of antibiotic X.

You have 3 sets of data to analyse for Experiment 5:

* antibiotic X-treated wild-type *E. coli* cells (TASK 5A)
* a time course showing wild-type *E. coli* cells after antibiotic X treatment (TASK 5B)
* mutant (antibiotic-resistant) *E. coli* cells treated with antibiotic X (TASK 5C)

TASK 5A-C. Microscopy of antibiotic-treated cells.

1. You have been provided with a set of data for each task (available on MyPlace).
2. You should also download the program ImageJ (instructions available on MyPlace).
3. Open each image in ImageJ.
4. You may wish to rotate or zoom to adjust the image.
5. Set a scale bar.
6. Measure cell lengths in μm.
7. Determine the best way to present your data. Are any statistical tests needed? How will you format and present any images?
8. How do these data compare with the data that you acquired in the lab?

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| Experiment 5 Discussion Questions |
| * We used microscopy to determine the effect of the unknown antibiotic “X” on *Candida albicans* and on *Escherichia coli* strains (both wild-type *E. coli* and an X-resistant strain). What conclusion(s) can you draw based on the results of this experiment? * Are there any other techniques you could have used to determine the effect of antibiotic “X” on *E. coli*? on *C. albicans*? * What conclusions can you draw based upon all of the experiments that you have performed (labs and workshops)? * What further experiments might you do (and why?)  45. Additional Resources/Further Reading for Experiment 545.1 Microscopy  * [Historical contributions from light microscopy: What can you learn with a light microscope?](https://www.ibiology.org/talks/light-microscopy/) * Cromey DW. Digital images are data: and should be treated as such. Methods Mol Biol. 2013;931:1-27. doi:10.1007/978-1-62703-056-4\_1 * Hartig SM. Basic image analysis and manipulation in ImageJ. Curr Protoc Mol Biol. 2013;Chapter 14:Unit14.15. doi:10.1002/0471142727.mb1415s102  45.2 Cell Shape  * Yang, Desirée C et al. “Staying in Shape: the Impact of Cell Shape on Bacterial Survival in Diverse Environments.” Microbiology and molecular biology reviews : MMBR vol. 80,1 187-203. 10 Feb. 2016, doi:10.1128/MMBR.00031-15 * Subashchandrabose S, Mobley HLT. Virulence and Fitness Determinants of Uropathogenic *Escherichia coli*. Microbiol Spectr. 2015;3(4):10.1128/microbiolspec.UTI-0015-2012. doi:10.1128/microbiolspec.UTI-0015-2012 * Cylke C, Si F, Banerjee S. Effects of antibiotics on bacterial cell morphology and their physiological origins. Biochem Soc Trans. 2022;50(5):1269-1279. doi:10.1042/BST20210894 |

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