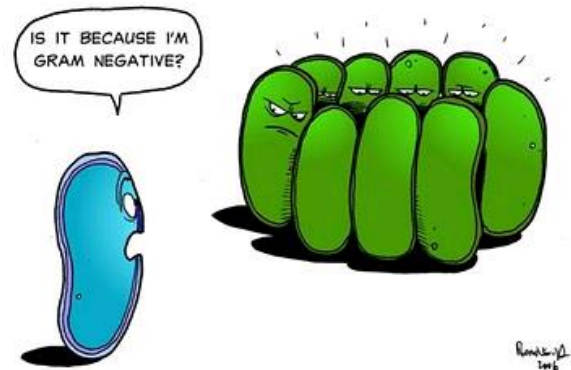**MICROBIOLOGY**

LAB SESSIONS

ARA GROUP 2013-2014

PROGRAMME

1.- Culture media

2.- Microorganisms are everywhere

3.- Laboratory culture: inoculation, incubation, and isolation of microorganisms.

The "aseptic technique".

4.- Sterility and contamination

5.- Measuring bacterial growth

6.- Effects of antibiotics and aeration on microbial growth.

7.- Bacterial identification.

8.- Antibiogram and antimicrobial agents

9.- Bacteriophage infection

INTRODUCTION

Through these lab sessions the students will become familiar with some of the basic techniques used in microbiology labs and will have the opportunity to check some of the aspects discussed during the theory lectures by themselves.

LAB SAFETY RULES

Although the microbes that will be used in the lab sessions are not normally pathogens, they still could cause problems if ingested or in contact with wounds, therefore they have to be handled as if they were pathogens. In addition, as we will be working with fire and flammable liquids (e.g. ethanol), we must be extremely careful, specially to prevent burning hair, clothes, etc. that could result is serious damage.

The following **safety rules** must be followed while in the microbiology lab:

• Open wounds must be covered. There are bandaids available in the lab's first-aid kit.

• Eating, drinking, chewing gum and/or smoking are **absolutely forbidden** in the lab.

• The use of mobile phones, tablets, and/or personal computers is **not allowed** in the lab unless with the explicit permission from the professor in charge.

• A lab coat must be worn during the lab session.

• Clothes and personal belongings cannot be left on the working benches.

• Before leaving the lab, students must leave their working space clean and tidy.

• Hands **must** be washed carefully before leaving the lab.

• Bunsen burners and alcohol must be handled with **extreme care**.

• Any incident with fire, cultures, glass objects, etc., must be reported **immediately** to the instructor.

1. CULTURE MEDIA

Culture media are the nutrient solutions used to grow microorganisms in the laboratory and, therefore, have to include all the nutrients needed for the growth of the microbes of interest. Culture media can be classified according to different characteristics. Thus, there are solid (with agar) or liquid (without solidifying agent) media, complex (thoseof which the exact chemical composition is unknown) and synthetic (with defined chemical composition), selective (appropriate for the growth and identification of a given microorganism), test (for biochemical tests) or enrichment media (that favor the growth of a group of microbes)

During this session we will prepare most of the culture media used throughout the rests of the lab sessions, including solid, liquid, test, selective, complex and enrichment media.

O/F (Hugh-Leifson) solid medium

1. Using a cylinder, measure 75 ml of distilled water and pour into a 100 ml flask.

2. Add 0.75g Hugh-Leifson medium.

3. Stir

4. Cover with a cotton cap and aluminum foil and sterilize by autoclaving.

5. Cool down to 60ºC.

6. Add 1 ml of a solution of 10% glucose previously filter-sterilized.

7. Aliquot in 5-ml portions (at least 14 tubes).

Gliding agar

1. Using a cylinder, measure 300 ml of distilled water and pour in a 500 ml flask.

2. Add 7.5g nutrient broth.

3. Dissolve by stirring.

4. Add 0.84g agar.

5. Cover with a cotton cap and aluminum foil.

6. Sterilize by autoclaving.

7. Cool down to 60ºC.

8. Stir and distribute into Petri dishes (at least 14 plates).

Solid Kligler medium

1. Using a cylinder, measure 200 ml of distilled water and pour in a 500 ml flask.

2. Add 11.6 g Kliger medium

3. Stir and melt in the autoclave.

4. Cool down to 60ºC.

5. Aliquot in tubes (approx. 10 ml/tube; at least 14 tubes).

6. Cover with aluminum caps and sterilize by autoclaving. After autoclaving, the tubes must be left tilted ("slants") so they can be inoculated appropriately (by stabbing through the center of the agar to the bottom of the tube and then by streaking the surface of the slant).

7. Let the tubes cool down and solidify.

Simmons citrate agar

1. Using a cylinder, measure 200 ml of distilled water and pour in a 500 ml flask.

2. Add 4.8 g Simmons citrate agar.

3. Stir and melt in the autoclave.

4. Cool down to 60ºC.

5. Aliquot in tubes (approx. 10 ml/tube; at least 14 tubes).

6. Cover with aluminum caps and sterilize by autoclaving. After autoclaving, the tubes must be left tilted to get a wide streaking surface.

7. Let the tubes cool down and solidify.

Nitrate broth

1. Using a cylinder, measure 60 ml of distilled water and pour in a 100 ml flask.

2. Add 0.56 g nutrient broth.

3. Stir until completely dissolved.

4. Aliquot in tubes (approx. 3 ml/tube; at least 14 tubes).

5. Cover with **black** aluminum caps.

6. Sterilize by autoclaving.

Tryptophan broth

1. Using a cylinder, measure 60 ml of distilled water and pour in a 100 ml flask.

2. Add 1 g Trp broth.

3. Stir until completely dissolved.

4. Aliquot in tubes (approx. 3 ml/tube; at least 14 tubes).

5. Cover with **red** aluminum caps.

6. Sterilize by autoclaving.

***Bacillus cereus* selective medium**

1. Using a cylinder, measure 300 ml of distilled water and pour in a 500 ml flask.

2. Add 12.9 g *Bacillus cereus* medium.

3. Stir.

4. Cover with a cotton cap and aluminum foil.

5. Sterilize by autoclaving.

6. Cool down to 60ºC.

7. Add one 15 ml egg yolk vial and one 1 vial of polymixin sulfate.

8. Distribute into Petri dishes (at least 14 plates).

M9 minimal medium

1. Using a cylinder, measure 230 ml of distilled water and pour in a 500 ml flask.

2. Add 4.8 g agar.

3. Cover with a cotton cap and aluminum foil. Sterilize by autoclaving.

4. Cool down to 60ºC.

5. Add 60 ml of sterile M9 salt solution.

6. Add 0.6 ml of sterile 1M $MgSO_4$.

7. Add 6 ml of sterile 20% glucose.

8. Add 0.15 ml of sterile $CaCl_2$.

9. Distribute into Petri dishes (at least 14 plates).

CGA medium (chloramphenicol glucose agar)

1. Using a cylinder, measure 300 ml of distilled water and pour in a 500 ml flask.

2. Ad 12 g CGA medium.

3. Stir.

4. Cover with a cotton cap and aluminum foil. Sterilize by autoclaving.

5. Cool down to 60ºC.

6. Distribute into Petri dishes (at least 12 plates).

Mueller-Hinton solid medium

1. Using a cylinder, measure 300 ml of distilled water and pour in a 500 ml flask.

2. Add 11.4 g medium

3. Stir

4. Cover with a cotton cap and aluminum foil. Sterilize by autoclaving.

5. Cool down to 60ºC.

6. Distribute into Petri dishes (at least 14 plates).

LC (Luria, Adams and Ting) medium without glucose

1. Using a cylinder, measure 500 ml of distilled water and pour in a 1l flask.

2. Add 5 g tryptone.

3. Add 2.5 g NaCl.

4. Add 5 g yeast extract.

5. Add 8 g agar.

6. Cover with a cotton cap and aluminum foil. Sterilize by autoclaving.

7. Cool down to 60ºC.

8. Add 2.5 ml of 1M $CaCl_2$ H_2O.

9. Add 2.5 ml of 1 M $MgSO_4$ $7H_2O$.

10. Stir.

11. Distribute into Petri dishes (at least 14 plates).

Nutrient agar

1. Using a cylinder, measure 300 ml of distilled water and pour in a 500 ml flask.

2. Add 9 g nutrient agar.

3. Stir.

4. Cover with a cotton cap and aluminum foil. Sterilize by autoclaving.

5. Cool down to 60ºC.

6. Distribute into Petri dishes (at least 14 plates).

2. MICROORGANISMS ARE EVERYWHERE

In this session we will observe and isolate some of the microbes that normally inhabit our skin and throat, the air surrounding us, the soil and the surfaces of common objects such as mobile phones, computers, the lab benches, etc… We don't intend to carry out a deep characterization of these environment microbiotas so we will observe only a small part of the microbes living in these habitats. Man,y of the microbes present in the analyzed samples may grow poorly, or not at all, on agar surfaces. Maybe the nutrients are not appropriate or the O_2 tension too high. We will observe mainly strict or facultative aerobes that do not require very specific or complex nutrients, as this is the kind of microbes that grow on nutrient agar (which is not an especially rich medium). Other factors that will be limiting the growth of microbes are pH and osmotic pressure, which could be different in the culture medium than in the natural environment.

First, we will isolate the microbes and then we will study their morphology, mobility and Gram staining. In addition, we will also use minimal medium as well as some selective media to see how different media retrieve different portions of the microbial diversity.

2.1 Skin and throat

Procedure:

1. Wash the skin between two fingers with soap and water (to remove the oily layer that covers the skin and hampers the release of the microbes) and rub with a sterile swab. Shake the swab inside a tube with saline solution (0.9% NaCl) to release the skin microbes. Spread the sample with the wet swab over the surface of a petri dish containing nutrient agar. Spread thoroughly in order to get separated colonies. Incubate for 24-48 hours at 37ºC. (Remember that, according to Brock's Biology of Microorganisms, "colonies are visible masses of cells formed from the division of one or a few cells and can contain over a billion (10^9) individual cells".)

2. Repeat the steps in point 1 but now rubbing a piece of skin previously cleaned with diluted ethanol.

3. Take a sample from the throat using a new sterile swab (try to avoid the teeth) and spread it directly onto the nutrient agar surface. Incubate for 24-48 hours at 37ºC. .

4. Once the colonies have grown, observe the bacteria under the microscope.

2.2 Surface

Surface sampling is frequently used as hygiene control, including applications such as checking the performance of disinfectants. When samples are taken from fissures, corners, cracks and crevices, sterile swabs are used. In the case of flat surfaces that are easy to clean and disinfect, samples are taken with the so called RODAC ("Replicate Organism Direct Agar Contact") plates, filled with the appropriate contact medium.

Procedure for surfaces:

1. Open a RODAC plate and press over the surface of interest (it can be a lab bench, a mobile phone screen, a computer keyboard…)

2. Close the plate and incubate at 37ºC during 24-48 hours.

3. Once the colonies have grown, describe them and observe the bacteria under the microscope.

Procedure for corners, crevices, etc.:

1. With a sterile swab rub a corner or crack of the lab bench (or any other object).

2. Immerse the swab in saline solution (0.9% NaCl).

3. Spread the swab onto a nutrient agar plate.

4. Close the plate and incubate at 37ºC during 24-48 hours

5. Once the colonies have grown, describe them and observe the bacteria under the microscope.

2.3 Air

There are several techniques available to estimate the microbial load in the air. They can make use of filtration, impact or sedimentation. We will use a very simple sedimentation-based technique to estimate the microbial contamination in the lab air. This kind of techniques relies on the fact that particles suspended in the air sediment by gravity. These particles can be recovered over an adherent surface (such as agar on a Petri dish). This approach is not quantitative (this is its main drawback) since (i) the volume of sample remains unknown and (ii) the larger particles can be overrepresented. However, it does not require expensive equipment and it is very useful for preliminary estimations of microbial loads.

Procedure:

1. Leave a nutrient agar plate open for 2 hours in the place you choose to sample avoiding air currents.

2. Cover the plate and incubate at 37ºC for 24-48 hours.

3. Once the colonies have grown, count them and observe the bacteria under the microscope.

2.4 Soil and "environment"

We can use different culture media to favor the selective growth of a given bacterium or to retrieve different parts of the culturable microbiota from environmental samples. In addition, the same microbes can show different morphologies when grown in different media.

In this session, we will observe the selective growth of *B. cereus* from soil and "environment" samples and observe how the use of different culture media retrieves different types of colonies from these samples. The "environment" sample has been prepared in advance by the instructor just by leaving an open flask with nutrient broth open in the lab for a while and incubating as described above.

Procedure:

1. Inoculate (by streaking or spreading) soil and "environment" samples onto CGA, M9 and *B.cereus* selective media (a total of 6 plates must be inoculated)

2. Incubate at 37ºC for 24-48h all the plates except CGA that has to be incubated at 30ºC for 48h.

3. Compare the colonies obtained in the different media and in point 2 of this session ("air")

3 LABORATORY CULTURE: INOCULATION, INCUBATION, AND ISOLATION OF MICROORGANISMS. THE "ASEPTIC" TECHNIQUE.

The goal of this session is to learn the most basic techniques of microbiology: inoculation, isolation and bacterial counting. These techniques allow us to have the bacteria amenable to study and obtain pure cultures from mixed cultures (the way bacteria are normally in nature)

CULTURE INOCULATION AND BACTERIAL ISOLATION

The goal of this session is to get isolated colonies from a bacterial culture. This will allow us to study the bacteria present in a culture and to obtain pure cultures from mixed ones.

Procedure (Note: always work within 10 cm of the Bunsen burner to avoid contaminations)

• Streaking ("agotamiento"). First, sterilize the inoculation loop in the flame (it must turn red) and let it cool down shortly close to the flame; open the tube containing the culture, introduce the loop, take a sample (avoid touching the tube walls) and recap the tube. Before and after taking the sample, flame the mouth of the tube. During this process do not leave the tube cap on the bench. Open a petri dish containing nutrient agar slightly and touch the agar surface with the loaded loop gently. Streak it through the medium surface spreading the sample in a zigzag motion (try not to break the agar…). Close the plate and incubate it upside down.

•Triple streaking ("estría escocesa"). Proceed as in the previous section but spread the sample as indicated in the figure below. After the first streak, close the plate,

flame the loop (to kill the remaining microbes), make sure it cools down and then, crossing the end of the first streak, make a second streak. Repeat this process three or four times. The goal is to get a very small amount of bacteria at the end of the streak so isolated colonies can be formed.

Once inoculated, incubate the plates (always upside down) in the oven at 37° C for 24 hours.
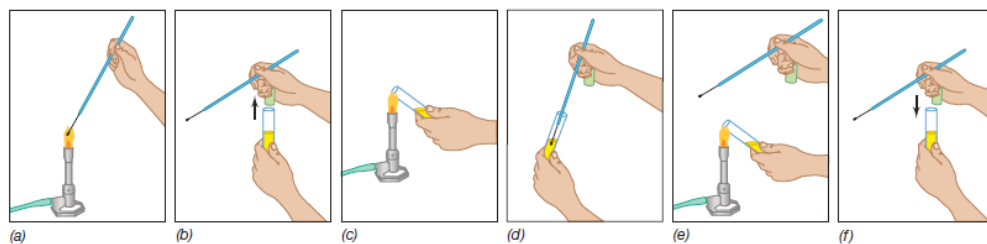
Figure 4.4 Aseptic transfer. *(a)* Loop is heated until red hot and cooled in air briefly. *(b)* Tube is uncapped. *(c)* Tip of tube is run through the flame. *(d)* Sample is removed on sterile loop for transfer to a sterile medium. *(e)* The tube is reflamed. *(f)* The tube is recapped. Loop is reheated before being taken out of service.
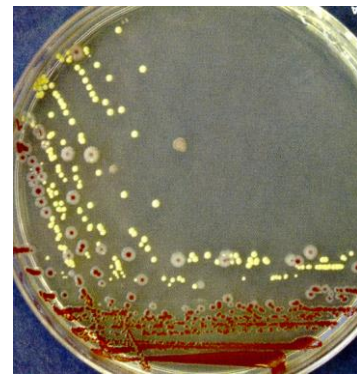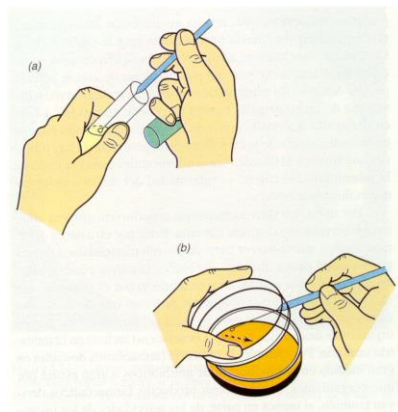
INCUBATION TECHNIQUES

There are several factors that affect bacterial growth. Among them, aeration and temperature are especially relevant.

Aeration: according to their O_2 requirements, bacteria can be:

- Aerobes: they need O_2 and depend on it for their growth.

- Facultative aerobes: they use O_2 when available but do not need it for their growth.

- Anaerobes: they cannot use O_2.

- Strict anaerobes: O_2 is toxic for them.

-Aerotolerant anaerobes: they do not use O_2 but can stand it.

- Microaerophiles: aerobes requiring low O_2 concentrations.

Depending on the type of bacteria of interest, there are several methods to provide more or less aeration. Normally, liquid media allow a better O_2 diffusion to the lower layers than solid media.

To incubate bacteria under aerobic conditions several approaches can be used:

- Liquid media: tube (tilted or in a shaker) or flask (with little medium, shaking and/or bubbling)

- Solid media: tube (slant) o plate (surface).

Anaerobic conditions are normally more difficult to achieve although the following systems can be used for culturing anaerobes:

- Liquid medium: standing tube (with sodium thioglycolate).

- Solid medium: tube ("Pre-Reduced, Anaerobically Sterilized") o plates (incubated in anaerobic jar or anaerobic oven).

Bacteria very sensitive to O_2 require extremely anaerobic conditions that can only be achieved with special equipment.

Temperature: Normally bacteria grow under a temperature range in which the following "cardinal temperatures" can be defined:

- Maximum: above this temperature bacteria do not grow and can even die.

- Optimum: at this temperature bacteria have their fastest growth.

- Minimum: below this temperature bacteria do not grow but normally do not die.

According to their optimum temperature bacteria can be:

- Hyperthermophiles(>70ºC)

- Thermophiles (40-70ºC)

- Mesophiles (10-50ºC)

- Psycrophiles (0-20ºC)

There are several ways to attain a constant incubation temperature:

- Oven: the temperature oscillations are normally within 1 to 3 degrees.

- Bath: The oscillations are normally smaller than 1 degree and therefore it is a better way to keep the incubation temperature stable.

Procedure:

1. Effect of the incubation temperature on the bacterial growth

We will inoculate three nutrient agar plates with the "environment" culture, using any of the techniques described above. One plate will be incubated at 55ºC, the second at room temperature and the remaining plate at 4ºC in the refrigerator After 24 hours, compare the colonies obtained in these plates with the ones previously inoculated and incubated at 37ºC and determine the type of microbes present in the inoculum.

2. Effect of oxygen on bacterial growth

We will inoculate two nutrient agar plates with a soil suspension, using any of the techniques described above. Prepare an anaerobic jar following manufacturer instructions and introduce one of the plates. Incubate both plates at 37ºC for 48 hours and compare the growth obtained in them.

4: STERILITY AND CONTAMINATION

Sterility is the absence of microbes (good or bad). Thus, sterile means free of microbes. Contamination is the opposite, that is, presence of microbes.

Disinfection is the elimination the pathogenic microbes present in a material by means of chemical agents or germicides. Notice that disinfestation may not eliminate all the microbes present in a sample.

Sterilization methods

- Physical agents:

Heat:

    Moist:

        Saturated steam (autoclave)

        Fluent steam

        Tyndallization

    Dry:

        Flaming

        Pasteur oven

Filtration

Radiation:

        Gamma rays

        UV light

Chemical agents:

        Ethylene oxide

        Glutaraldehyde

Disinfection methods

Alcohols:

ethanol

isopropanol

phenol

Boiling

Procedure:

We will try several methods using the "environment" culture:

1. **UV light**

Spread 0.1 ml of inoculum over one nutrient agar plate using a glass rod. Open the lid and cover half of the plate with aluminum foil. Put the plate under the UV lamp. Each table will irradiate a plate for a given period of time, so the effect of the irradiation time can be observed. After the irradiation, cover the plate and incubate at 37ºC for 24 hours.

2. **Boiling**

Boil the remaining "environmental" sample by heating the bottom of the tube over the Bunsen burner. Keep the tube **open** and shake gently! When the liquid starts to boil, take the tube away from the flame and wait for a short while. Repeat this procedure three or four times and let the liquid cool down. Then spread a new plate with 0.2 ml of inoculum using a glass rod. Incubate at 37ºC for 24 hours.

Boiling does not sterilize as it eliminates vegetative forms but not endospores. Therefore, we will keep this plate (if colonies have developed) to carry out an endospore staining (see below).

5. MEASURING BACTERIAL GROWTH

The number of bacteria in a population can be determined using either direct or indirect methods such as the estimation of cell mass. Optical density (OD) readings are very frequently used to estimate cell concentrations in cultures once the absolute number of cells has been determined by a direct method such as CFU counting or direct counting of cells under the microscope. In any case, OD readings are very useful to monitor growth and the changes in cell density, even if the absolute cell numbers remain unknown.

CFU counting

This technique has two goals: (i) to get isolated colonies and, (ii) to estimate the colony forming units (CFU) present in a given sample. For this purpose, we first must obtain, by diluting the initial sample, a bacterial solution with the appropriate concentration. In other words, the concentration of bacteria in this solution must produce, after spreading onto a plate, a countable number of colonies (not confluent growth). A number of 30-300 CFU per plate is considered statistically valid.

Direct counting

The total number of cells (or "total counts") present in a microbial suspension can be determined by direct microscopy observation. Three are several ways for counting microbes under the microscope using fresh or stained samples. In this session we will use a Thoma cell counting chamber. This is a special microscopy slide that contains a large central square divided into smaller squares (see figure).

Procedure:

Inoculate 5 ml of an overnight culture of *Escherichia coli* 50 ml of LB medium enriched with 0.2% (w/v) glucose. Incubate in the shaker at 37ºC. Take 2 ml samples at different times and keep on ice until used. For every sample, take an OD reading, make a serial dilution bank and count cells in the counting chamber.

1. Optical density: read the optical density of the culture at 600 nm (OD_{600}). Optical density is proportional to cell mass (according to the Lambert-Beer law). This proportionality is lost for OD values close to 1. Therefore, when values above 0.8 are obtained, the sample must be diluted with the appropriate volume of sterile LB medium For instance, dilute 1 ml of culture with 4 ml of LB. The corresponding OD_{600} multiplied by the dilution facto (x5) will be proportional to the cell mass concentration in the culture.

2. Serial dilutions: For this experiment we will need 1 tube with 9.9 ml of saline solution (0.9% NaCl) and 4 tubes with 9 ml. With a sterile pipette, transfer 0.1 ml of culture to the 9.9 ml tube (tube number 1). Shake the tube to homogenize the bacterial suspension and with a new pipette transfer 1 ml from this tube to a second tube with 9 ml of saline solution (tube number 2). Shake again and, always using a new pipette, now transfer 1 ml to a new tube (number 3); repeat the procedure transferring from tube 3 to tube 4 , 4 to 5, and 5 to 6 (remember that all tubes, except number 1, contain 9 ml of saline solution). Then, with a glass rod, spread 0.1 ml from al the dilutions into the corresponding nutrient agar plates, as follows: flame the mouth of a sterile pipette, get at least 0.2 ml of bacterial suspension, uncap the plate slightly and transfer 0.1 ml of the suspension onto the medium surface. Dip the glass rod in alcohol, carefully and away from the ethanol container, flame the glass rod shortly and, away from the Bunsen burner, leave the flame to consume the alcohol. Then, cool the glass rod down, and opening the plate (do not open it completely and keep the cover over the plate!), spread

evenly the bacterial suspension over the medium surface. Recap the plate, leave it upside down and incubate it at 37ºC for 24 hours.
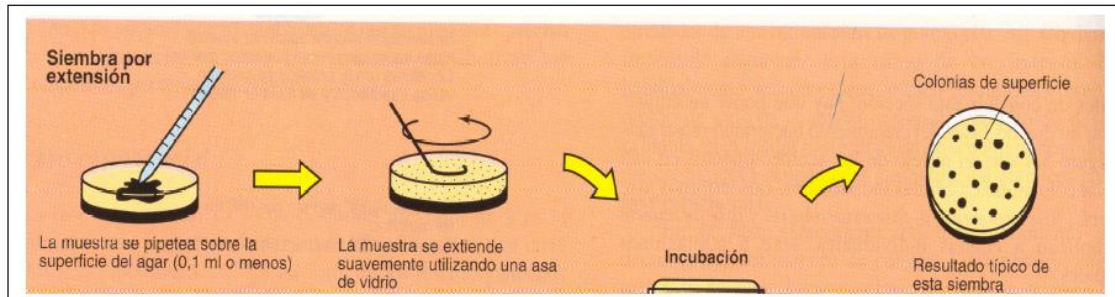

After the incubation, count the colonies and calculate the bacterial concentration in the original tube (this number is normally expressed as CFU/ml rather than cell/ml, why?). For this calculation, you have to consider the amount of inoculated sample (0.1 ml) and the dilution factor.
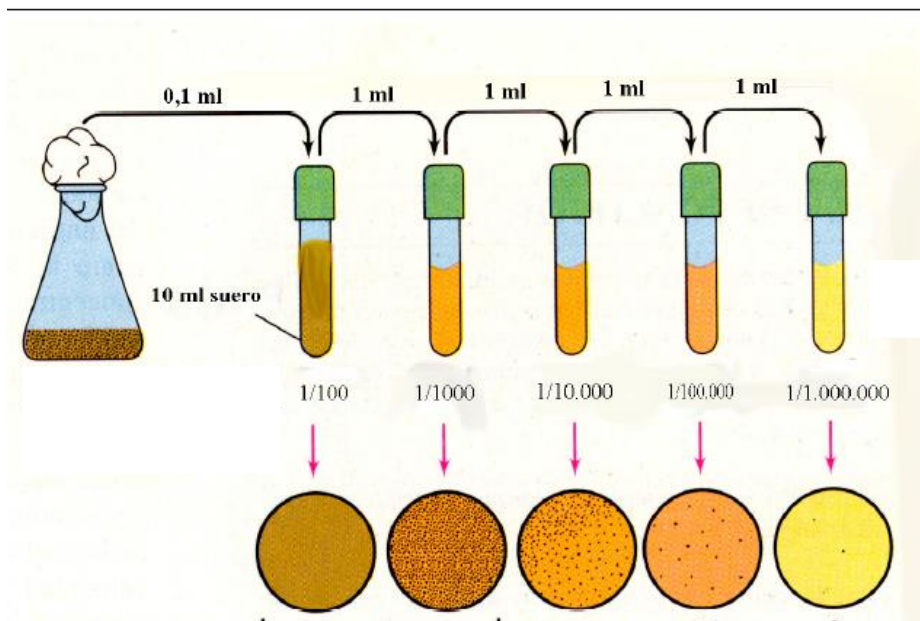
3. Microscopy counts: using the serial dilutions prepared above and using a micropipette, fill a counting chamber by capillarity by letting the pipette tip lean softly on the edge between the cover slide and the chamber. Observe the preparation under the microscope. First, use the 40X objective to locate the grid and the use the 100X objective to count the cells. Count the microbes inside the squares located in the two

0,25 mm   0,05 mm
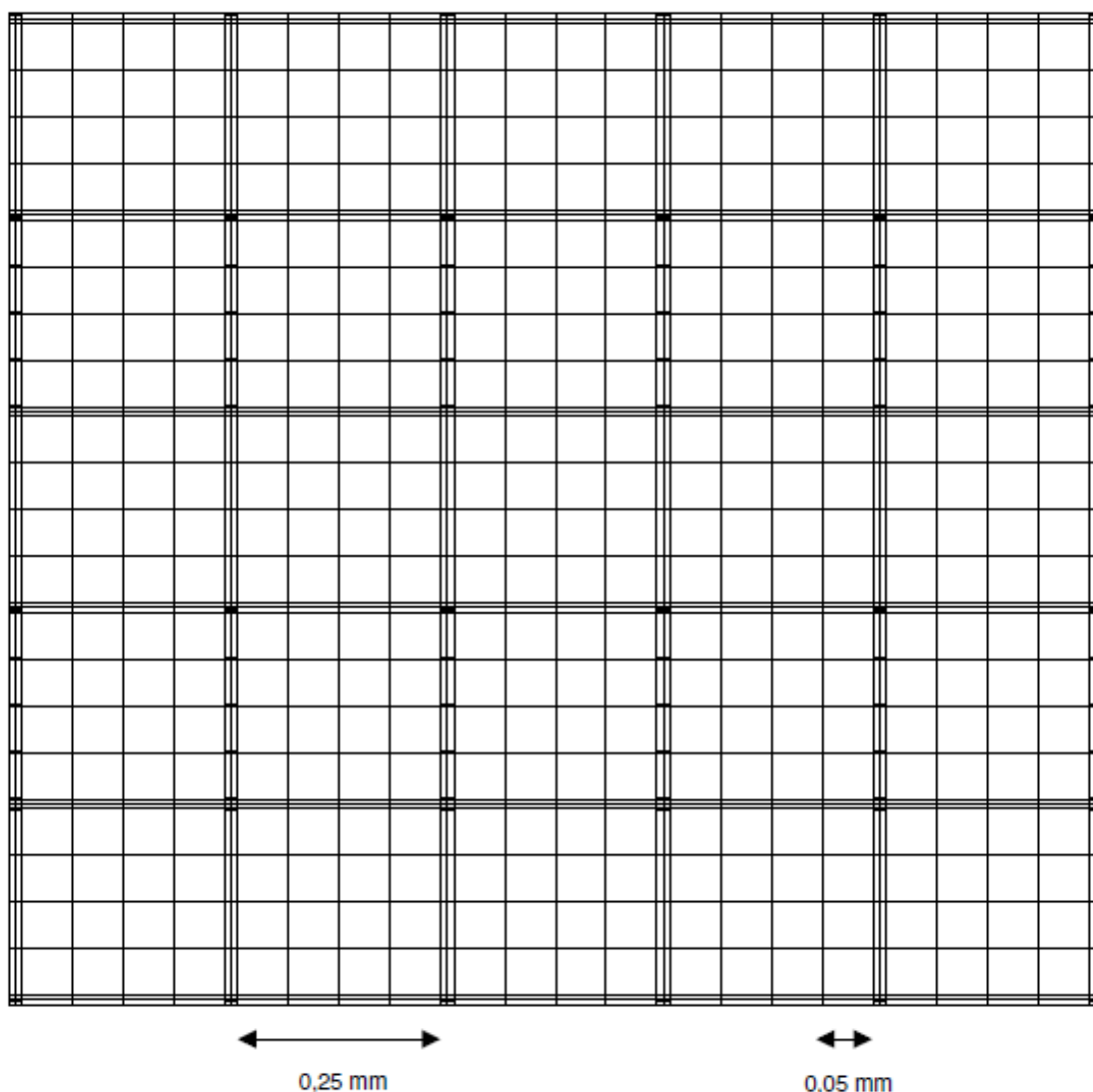
diagonals (40 squares in total). Each square side is 0.05 mm long and thus, the square

area is de 0.0025 mm2. The corresponding liquid volume is 0.00025 mm^3. $=.2.5 \times 10^{-4}$

mm$^3 = 2.5 \times 10^{-7}$ml. Thus, the bacterial concentration in the sample will be:

Microbes/ml = counted cells / (40 x 2.5×10^{-7} x dilution factor)

6. EFFECTS OF ANTIBIOTICS AND AERATION ON MICROBIAL GROWTH

In this session, we will analySe the growth kinetics of an *E. coli* culture and

observe how antibiotics and aeration affect it. We will use two antibiotics frequently

used to treat bacterial infections: ampicillin and streptomycin.

Procedure:

Using the aseptic technique, inoculate 1 ml of an overnight culture of *E. coli* into

a flask containing 75 ml of LB medium enriched with 0.2 % (w/v) glucose. Mix it,

transfer 1 ml to a spectrophotometer cuvette and immediately bring the flask to the water bath shaker or to the oven (as assigned to your group). Keep record of the exact time at which you take the initial sample (this will be "time zero"). Try to keep the cultures at the same temperature during the whole experiment as, if they get colder, the growth will slow down. Take samples every 25 minutes (make sure you write down the time at every sampling point).

For every sample, measure OD600 (dilute when above 0.8 as explained above) and keep a record of the obtained values as you will have to draw a growth curve. When the cultures reach OD of 0.35-0.5, add the antibiotic (see table) and keep taking samples every 25 minutes until the end of the session.

	Incubation	Antibiotic (when OD between 0.35 and 0.5)
BENCH 1	37ºC WITHOUT shaking	None
BENCH 2	37ºC WITH shaking	None
BENCH 3	37ºC WITHOUT shaking	0.1 mg/ml ampicillin
BENCH 4	37ºC WITH shaking	0.1 mg/ml streptomycin

7. BACTERIAL IDENTIFICATION

To identify bacteria several assays can be used, including microscopy observation and biochemical tests. Observation of microbes under the light microscope provides information on their shape, motility, presence of specific structures such as endospores, and cell envelope composition (by using Gram staining for the peptidoglycan, or Zeel-Nielsen's for mycolic acids). Biochemical tests are carried out to

analyse the metabolic characteristics of pure cultures. Before the advent of molecular biology, bacterial taxonomy relied mainly on microscopy and biochemical tests, although now molecular tests are also used for bacterial identification. In any case, the kinds of methods that we will learn during this session are still widely used in different fields of microbiology.

Each table will be given an "unknown" bacterium that will have to be identified using microscopy and the biochemical tests explained below. For this purpose, an identification key is provided at the end of the explanations.

7.1.- BIOCHEMICAL TESTS

Pigment production: the goal is to ascertain whether your unknown bacterium produces pigments or not.

Procedure: spread the unknown bacterium on an agar nutrient plate. Incubate at 37ºC for 24 hours.

Catalase assay: we investigate the presence of catalase, an enzyme able to split H_2O_2 into H_2O and O_2 (with the subsequent production of bubbles).

Procedure: take a loopful of bacteria from a solid culture, place it over a microscope slide, add a few drops of 10 volume hydrogen peroxide 10V and see whether bubbles appear or not.
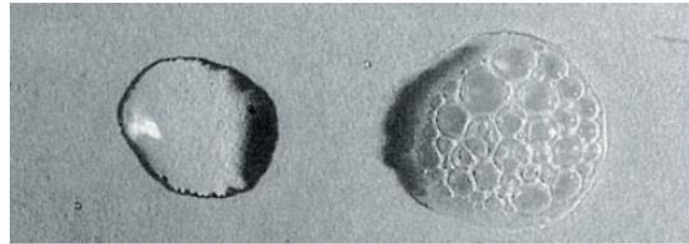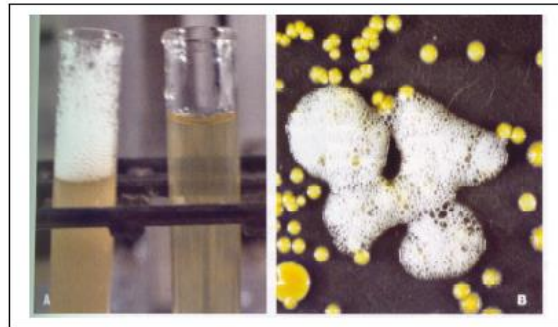
Figure 5.30 Method for testing a microbial culture for the presence of catalase. A heavy loopful of cells from an agar culture was mixed on a slide (right) with a drop of 30% hydrogen peroxide. The immediate appearance of bubbles is indicative of the presence of catalase. The bubbles are O_2 produced by the reaction $H_2O_2 + H_2O_2 \rightarrow 2\,H_2O + O_2$.

Oxidation-fermentation assay (O/F): sugars can be metabolized either by the oxidative (respiratory) or the fermentative pathway. In the oxidative pathway, the final electron acceptor is the oxygen. This process, obviously aerobic, does not produce a strong decrease in the medium pH (i.e. acidification). On the contrary, in the fermentative pathway the final acceptor is an organic compound derived from the metabolized sugar. This process is anaerobic and produces a fast and sharp acidification of the medium. To ascertain which of these pathways are used by our unknown bacterium we will use two tubes of glucose containing medium, one aerobic (with an air chamber) and the other anaerobic (covered with a paraffin layer), as well as a pH indicator that will change the colour of the medium from green to yellow if acidification occurs.

Procedure: inoculate two tubes of Hugh-Leifson medium by stabbing the loop as deep as possible. Cover one medium surface with a small amount of sterile paraffin and

incubate both tubes at 37ºC for 24 hours. The medium will turn yellow according to the metabolism of the bacterium (only slightly the upper part of the open tube, on the bottom of both tubes, along the whole medium, etc…). With these clues, you will find out the metabolism of your unknown bacterium

Acid and gas from carbohydrates using Kliger-Iron medium: this assay is used to check whether a bacterium ferments a carbohydrate producing gas and/or acid.

Procedures: inoculate Kliger tubes by stabbing the loop in the middle of the slant until the bottom of the tube and, when taking the loop out of the agar, streak it on the agar surface with a zig zag motion. This double inoculation allows growth of your unknown organism on the aerobic surface of the slant and in the largely anaerobic butt of the tube. Incubate at 37ºC for 24 hours.

This is a complex medium that contains a large amount of lactose and a very small amount of glucose; a pH indicator (yellow in acid and red in base); and iron, which is precipitated as a black sulfide if H_2S is produced (see below). These ingredients allow you to determine four biochemical properties of your unknown organism:

- Lactose (+) or (-). At the surface of the slant (aerobic conditions), only fermentation of carbohydrates present in high concentration (in this case lactose) yield

more acidic products than can be oxidized to neutrality. Thus lactose (+) organisms yield a yellow slant and lactose (-) organisms yield a red slant.

- Glucose (+) or (-). In the largely anaerobic butt of the tube, even fermentation of the trace concentrations of glucose yields enough acid to change the pH. Thus, glucose (+) organisms yields a yellow butt. Fermentation of lactose in the butt will obviously also change the pH, but this does not confuse the interpretation since all lactose-positive organisms are also glucose-positive. If the tube gets black from iron sulfide (see below), you can presume the underlying colour is yellow.

- Gas formation (+) or (-). If an organism forms gas from glucose or lactose the agar in the butt will show bubbles or cracks.

Sulfhydric production (Kligler): bacterial activity on sulfur-containing aminoacids frequently releases suflhydric acid. This compound can react with metallic salts such as iron salts contained in the Kliger agar) and forms a dark precipitate.

Procedure: observe the Kliger agar after incubation (see above). If your unknown bacterium forms H_2S, the lower portion of the tube will turn black, due to the

formation of iron sulfide. Do not confuse a pigment associated with colony growth on the slant with the black ferric sulfide in the butt.

Use of citrate as carbon source: this test is used to see if our unknown bacterium is able to grow with citrate as the sole carbon source and ammonia salts as the nitrogen source. We will use the Simmon's citrate agar medium that contains a pH indicator that turns blue at alkaline pH (this happens when the citrate is used, and the medium changes from green to blue).

Procedure: inoculate the tubes by spreading the loop over the agar surface. Incubate at 37ºC for 24 and observe the colour of the medium.


Nitrate reduction: with this test we check if the unknown bacterium reduces nitrate to nitrite. When nitrite is produced, a red colour develops after the addition of the appropriate reagents.

Procedure: inoculate a tube of nitrate broth with the unknown bacterium and incubate at 37ºC for 24 hours. Then add a couple of drops of each reagent (reagent A:

0.8% sulfaminic acid in glacial acetic acid; reagent B: 0.5% alfa-naftilamine in glacial acetic acid). If a pink colour develops, the test is positive

Indol production: some bacteria produce indol as a product of tryptophan degradation. Indol can be detected because it reacts with Kovacs reagent producing a deep pink colouration.

Procedure: inoculate the tryptophan broth with the unknown bacterium and incubate at 37ºC for 24 hours. Then add one drop of Kovacs reagent and let the tube standing for a short while. The test is positive if a pink ring develops in the interphase.
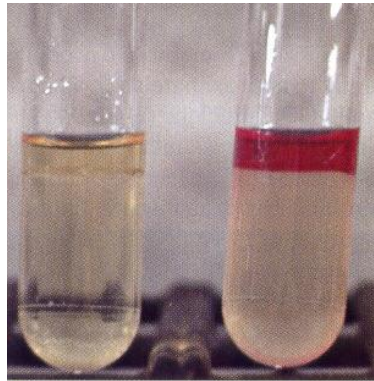
7.2.- MACROSCOPIC OBSERVATION

Macroscopic observation: although bacteria obviously are too small to be seen with the naked eye, the colonies they form are visible and provide useful information for bacterial identification. We can observe the size, shape, margin, elevation, pigmentation (distinguishing between pigmented colonies and those secreting diffusible pigments), texture (butyrous, viscous, or dry) and opacity (transparent, translucent, or opaque). There are smooth colonies with a shiny glistening surface; rough with dull, bumpy, granular, or matte surface; or mucoid of slimy or gummy appearance.
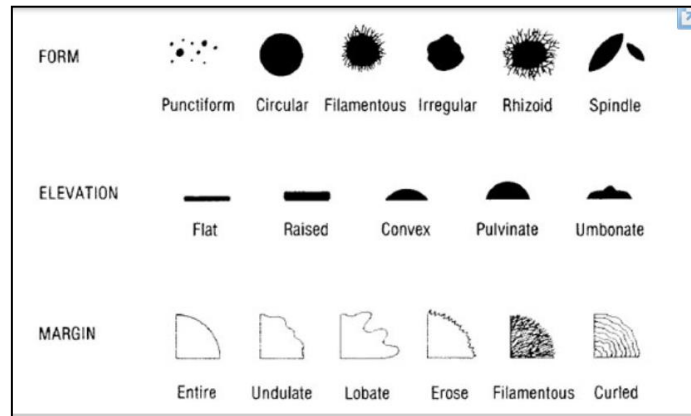
The aspect of liquid cultures is also useful for identifying microbes since they can modify the medium as a consequence of their metabolism. Thus, we can observe if bacteria produce an uneven turbidity, if they grow only on the interphase with air, if they produce pigments, etc.

Procedure: inoculate one nutrient agar plate with your unknown bacterium and observe the colonies according to the aspects discussed above.

Gliding motility on semisolid agar: most bacteria form colonies on solid agar because they cannot move on this kind of surfaces. However, when they are grown in semi-solid media (containing 0.28% agar) motile bacteria can move by finding their way in the agar network giving rise to diffuse colonies that can cover the whole agar surface.

Procedure: Using an isolated colony of your unknown bacteria, inoculate a gliding agar plate using a sterile toothpick. Never turn the plate upside-down! Incubate at 37ºC overnight. Radial growth from the inoculation point indicates gliding motility. Compare the diameter of the colonies produced by the different unknown bacteria.

8: ANTIBIOGRAM AND ANTIMICROBIAL AGENTS

8.1. ANTIBIOTICS

Procedure:

Inoculate a Mueller Hinton plate with a swab soaked in the culture of the unknown bacterium. First, draw a star in the centre of the plate and then spread the sample evenly to get confluent growth. Make sure all the surface becomes into contact with the inoculum.

With flame forceps, get the antibiotic disks from their containers and place them on the agar surface. Press the disk slightly against the surface so they remain adhered to it. Use different antibiotic disks and try to place them symmetrically on the agar surface. Incubate at 37ºC for 24 h and measure the diameter of the inhibition zones around the antibiotics and, using the corresponding table, assess the sensitivity/resistance of your bacterium to the assayed compounds.
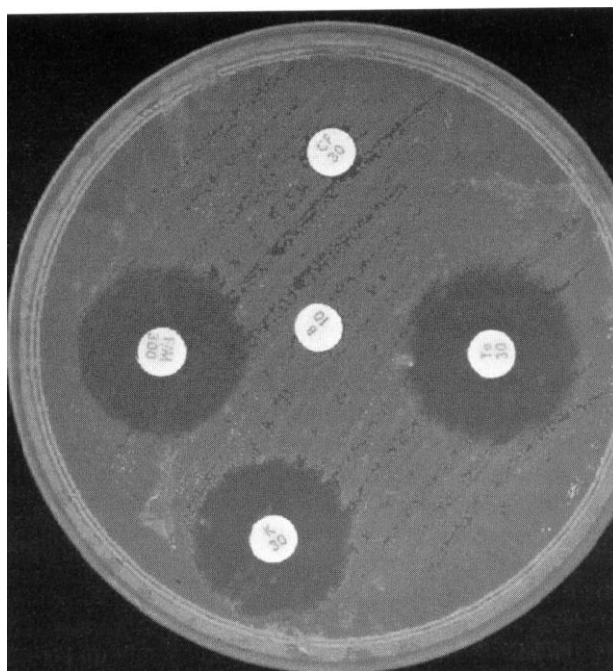
Antibiograms cannot be used for bacterial identification. Their goal is to ascertain whether a bacterium is sensitive or resistant to the antibiotics tested. As antibiotic resistance is a character frequently coded in plasmids, it can transfer between different bacterial strains, and even between different species, and therefore cannot be considered a taxonomic character or a conclusive character in bacterial identification. However, there is some specificity regarding antibiotic sensitivity in some bacterial groups.

8.2. GERMICIDES

Procedure:

Inoculate a nutrient agar plate with your unknown bacterium using a soaked swab as indicated above. Using flame forceps soak five paper disks into different germicides and let them dry over a Petri dish. Then, place them onto the inoculated plate and proceed in the antibiotic assay.

9: BACTERIOPHAGE INFECTION

Bacteriophages are viruses that infect bacteria; they are DNA and/or RNA molecules surrounded by a protein envelope. Some authors also call phages those viruses infecting *Archaea* (although they do not always display 'normal' phage-like behaviour and structure). For this reason, other authors propose the classification of viruses into three categories: archaeoviruses, bacterioviruses and eukaryoviruses. In any case, viruses infecting microbes seem to be the most abundant "biological entities" of

the planet, with an estimate of 10 viruses per prokaryote in the environment. Viruses play a very important role in controlling prokaryotic numbers and diversity, as well as favoring genetic interchange mediated by transduction.

Bacteriophages can be studied by different techniques that include classical isolation methods and culture-independent approaches such as metagenomics. As with prokaryotes, culture is a very powerful approach to characterize a phage although it has a very strong bias imposed by the well-known great plate count anomaly. When we culture bacteria, we look for colonies (a group of cells that come from the division of an isolated cell) although when we culture phage we look for the effect of the virus on its host. So, first, we must grow the host (normally by getting confluent growth) and look for lysis plaques where phage infection has occurred. (See the definition of plaque in the "Brock's Biology of Microorganisms": "when a virion initiates an infection on a layer of host cells growing on a flat surface, a zone of lysis may be seen as a clear area in the layer of growing host cells. This clearing is called a plaque, and it is assumed that each plaque originated from the replication of a single virion".)

During this lab session we will infect *Escherichia coli* with bacteriophage P1. Two different host strains will be used: K12 MG1655 that has restriction-modification systems and strain BL21 DE3, which lacks these systems. We will carry out a plaque assay and compare the natural resistance to phage infection of both strains.
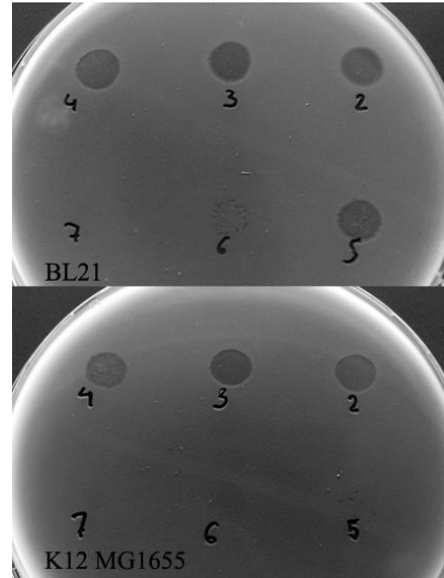
Procedure:

1. Prepare serial decimal dilutions of the P1 stock (vial P1) using sterilized P1 buffer.

2. Add 2.5 ml of TOP-LC medium to each culture tube (K12MG1655 and BL21). Vortex for 3-5 seconds and pour rapidly over a Petri dish plate containing LC medium.

3. Wait for 10 min for the agar to solidify, dry for 1 h at 37ºC and then spot regularly 5 μl of the dilutions 10^{-2} to 10^{-7} over the medium surface.

4. Once the spots have been absorbed into the medium, incubate at 37ºC for 24 h. Compare the efficiencies in plaque forming between the two infected strains.