MiSAC helps

PRODUCED BY THE MICROBIOLOGY IN SCHOOLS ADVISORY COMMITTEE

MiSAC helps give examples of responses to enquiries from teachers and technicians on various aspects of microbiology, particularly practical work.

Microscopy and staining
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Microscopy and staining

Why haven't we had much success with seeing bacteria in preparations stained by Gram's method?

The problem is more likely to lie with preparation of the smear than with the staining procedure. More success is obtained by taking growth from the surface of a culture grown on an agar medium, e.g. nutrient agar, than from a liquid culture medium, e.g. nutrient broth.

The reason for this is two-fold. (1) Smears prepared from a broth culture medium tend to be washed off the slide during the staining procedure because the proteinaceous content of the medium transferred to the smear interferes with adherence of bacteria to the microscope slide. A smear prepared from growth on the surface of an agar culture medium adheres better because culture medium is not carried over to the smear. (2) The density of bacteria in a broth culture is often less than ideal for making a smear that contains an adequate number of bacteria.

It is also essential to prepare a smear which is evenly mixed and is not so dense that the shape and arrangement of individual bacteria cannot be distinguished. After drying the smear, either by allowing it to dry naturally or by gentle warming over a Bunsen burner flame, 'fix' the smear to the microscope slide by passing it once or twice through the flame.

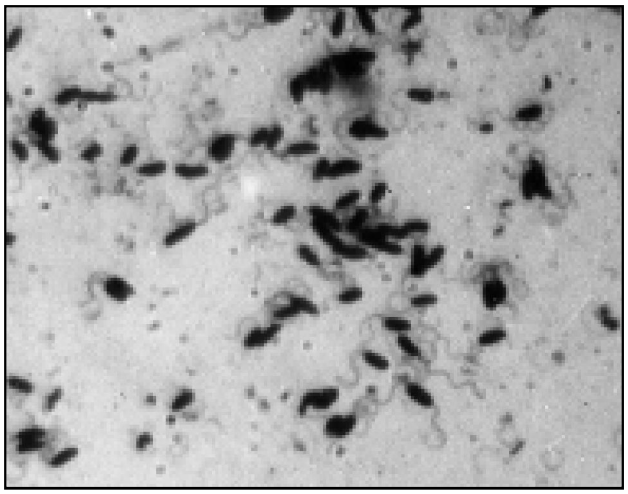
After completing the staining procedure and rinsing the slide under a running tap, it is essential to dry the smear thoroughly by shaking off remaining tap water and then completing the process by pressing filter paper or blotting paper onto the preparation. Residual water and immersion oil are immiscible and the droplets of water consequently formed cause reflection and refraction of light which make it impossible to focus on the specimen.

Which bacterial cultures are suitable for observing motility by the hanging drop method?

Pseudomonas fluorescens and *Eschericia coli* are examples of motile bacteria. *Ps. fluorescens* has polar flagella and is more rapidly motile than the peritrichously flagellated *E. coli*. *Micrococcus* spp. and *Staphylococcus* spp. are non-motile.

It is helpful to examine a non-motile culture to distinguish between Brownian movement, to which all bacteria are subject, and motility. Small particles of about 1 µm in dimension (the size of bacteria) suspended in liquid media make small movements because of being bombarded by the surrounding molecules of the medium. Such movements are random and the particles do not change position which is in contrast to motility by which bacteria travel up to 100 cell lengths per second. This is a remarkable phenomenon because for bacteria the viscosity of a watery medium is equivalent to that of syrup.

Bacterial flagella are too thin to be observed directly with an ordinary bright-field microscope unless stained using special techniques to exaggerate their thickness.



Pseudomonas fluorescens showing polar flagella (silver impregnation stain viewed with an oil-immersion objective lens).

HINT 1: A young culture, e.g. 24 hours old, in a liquid medium is recommended for observing motility. For the cultures named above, use either a nutrient broth or a nutrient agar slope to which has been aseptically added a few drops of sterile nutrient broth or sterile water before inoculation. Moist agar slopes have advantages in that particularly good motility occurs at the liquid-agar interface, there is also growth on the agar surface for making stained preparations (see comment on making preparations for staining by Gram's method), and they are less liable than broth cultures to accidental spillage.

HINT 2: The hanging drop method involves observing a sample of unstained culture on the underside of a cover slip suspended over the depression ('cavity') in a cavity

slide. The possibility of the sample of culture touching the bottom of the cavity and spoiling the preparation can be avoided by increasing the height of the cover slip in the following way. GMLP must be carefully observed because the preparation contains living bacteria.

- Place a small portion of Vaseline© on each of two diagonally opposite sides of the slide just beyond the cavity edge.
- Aseptically transfer a loopful of liquid culture to the centre of a cover slip.
- Invert the slide over the cover slip and lower it only enough for two corners to make light contact with the Vaseline© so that the slide and cover slip are attached together.
- Quickly invert the slide.
- Use the low power objective lens to focus on the edge the loopful of culture beneath the cover slip and then without altering the focus bring the high power dry objective lens into position. Increase the light intensity as necessary by opening the condenser iris diaphragm. Only a slight adjustment of the fine focus is usually necessary to see the bacteria because the different objective lenses are parfocal.
- Discard the preparation into disinfectant immediately after use because it contains living bacteria.

What method would you recommend for examining a hay infusion microscopically?

A wet mount preparation is appropriate for studying a hay infusion*. It has the advantage over a hanging drop preparation in providing a shallow film which requires only small adjustments of focus to keep the various organisms in view. The rate at which the preparation dries out from the effect of heat from the microscope lamp can be reduced by sealing the preparation.

The traditional method of sealing the preparation involves putting a sample within a ring of Vaseline© on a microscope slide and placing a coverslip on the ring. However, it is very difficult to prepare the ring neatly and therefore the following procedure should be considered. Remember that you are using living specimens which must be handled and disposed of safely.

Place 1 or 2 loopfuls of sample on a slide and cover with a coverslip, taking care to exclude air bubbles. Warm the end of another slide in a Bunsen burner flame, dip it into Vaseline© and immediately touch it along the full length of one edge of the coverslip. A thin film of cooled and solidified Vaseline© will seal the edge of the coverslip. Repeat the procedure for the other 3 edges of the coverslip.

Examine the preparation with a low power objective lens, e.g.x10. This provides an adequate magnification, i.e. x100 when used with a x10 eyepiece lens, for seeing protozoa, algae and small animals, i.e. rotifers and nematodes. It is most important to adjust light intensity to achieve the optimum balance between brightness and definition. Glare caused by the light being too bright is a common reason for being unable to see the specimen. If the microscope is fitted with a sub-stage condenser, optimum illumination is achieved by adjustment of the inbuilt iris diaphragm. With correct lighting, bacteria can also be seen which enables size comparisons with larger microbes to be made. If it is necessary to increase the magnification, turn the high power dry objective lens (x40) into position without moving the focus, increase the light intensity and then refocus. Only a small adjustment of the fine focus control is usually needed because the different objective lenses are parfocal.

NOTE:** Examination of natural pond water is rarely rewarding but looking at a hay infusion or dirty flower vase water is a simple and effective way of observing communities of living organisms and successions in their development. A hay infusion can be readily made by adding a palmful of chopped hay to ca100 cm^3 of rain, stream, pond or lake water in either a conical flask or medical flat bottle laid on its broad side. Provision of a shallow layer and closure with a non-absorbent cotton wool plug promote good aeration. Incubate at room temperature either on window sill away from direct sunlight or under artificial light. An extension of this activity to water pollution is given in MiSAC's *Practical Microbiology for Secondary Schools, pp18-19, which includes using 0.1% (w/v) phosphate and/or nitrate to demonstrate eutrophication caused by fertilizer run-off from farmland.

** Available free-of-charge from the publishers, the Society for General Microbiology (see Useful Links page for contact details).

Hay infusion in medical flat bottle closed with a non-absorbent cotton wool plug.

Media and culture methods

For how long are culture media useable after preparation?

Sterilized agar and liquid culture media can be stored for several months at room temperature if precautions are taken to prevent evaporation, ideally by dispensing in screw-cap medical flat bottles. Storage should be away from direct sunlight otherwise products inhibitory to growth may be formed. Before use always check for contamination of media.

Poured agar plates can be stored under the same conditions, base uppermost, in Petri dish bags tled with a knot or an elastic band to prevent evaporation. Again, check for contamination before use. Storage in a refrigerator is an unnecessary use of valuable space and condensation that forms in the plates has to be dealt with before use.

HINT 1: Bubbles in poured agar plates can be removed by quickly passing a Bunsen burner flame over the surface of the medium before it solidifies. The Petri dish lid must be raised as little as possible to prevent contamination.

HINT 2: Minimize condensation on the Petri dish lid by allowing molten agar media to cool to about 50ºC before pouring, Agar media solidify at 42-43ºC.

HINT 3: In exceptional circumstances, condensation can be removed quickly in a 50-55ºC oven. Holding the closed Petri dish vertically, open it over the oven shelf with the inside of the lid and surface of the agar medium pointing downwards. Place the lid on the shelf and the edge of the base supported on it. Leave for not more than about 10 minutes, otherwise the medium will begin to evaporate.

I would like to economise on the number of agar plates required for making colony counts. Is it there a technique for inoculating more than one dilution on a plate?

There is a well-established technique for doing this. It was devised in the late 1930s for use in hospital pathology laboratories and is ideal for use in schools but as yet not widely known there. A protocol is described in MiSAC's *Practical Microbiology for Secondary Schools* (pp 34-35) available free-of-charge from the publishers, the Society for General Microbiology (see Useful Links page for contact details).

The procedure involves inoculating separate sectors of the surface of an already poured plate of an agar culture medium with small measured volumes of dilutions of a sample, e.g. 0.02 cm^3 (20 µl). This is approximately the volume of one drop delivered from a vertically held Pasteur pipette. Each inoculum spreads over an area of about 1 cm in diameter, making it possible for 4-6 dilutions to be accommodated on a plate.

HINT 1: The technique is suitable for use with pure cultures of bacteria or yeasts that form discrete colonies, i.e. their growth does not spread. Fortunately this is a feature of the cultures listed as being suitable for use in schools. For this reason it also can be used with some natural samples, e.g. water, but is less successful with others, e.g. soil, which contain bacteria that form spreading growth which makes it difficult to distinguish individual colonies for counting.
HINT 2: Plates must not be inverted until the inocula are absorbed into the agar medium. This process can be hastened by using well-dried plates that have been poured 2 or 3 days in advance and incubated (even at room temperature) base uppermost.

HINT 3: Check the plates daily until separate, countable colonies are seen. It may be necessary to refrigerate the plates before the next practical class in order to arrest growth thereby prevening colonies from merging into each other. Alternatively, incubation at room temperature might allow growth to be slow enough to avoid the necessity for refrigeration.

/Investigations

Investigations

In a practical investigation for an Applied Science unit to simulate a hospital pathology laboratory scenario for identifying pathogens in a sample taken from a hospital patient, I would like to use a culture which would give the same results as *Escherichia coli* but without the danger of exposure to any pathogens.

It is acceptable to use the cultures of *E.coli* that are available from reputable schools suppliers, i.e. strains B and K12. These strains present minimum risk given that good microbiological laboratory practice (GMLP) is observed.

As part of a unit on micro-organisms and food safety, students need to be able to make streak plates to identify the types of bacteria present. How can the identification be made from examination of the various colony forms?

A nutrient agar streak plate from soil showing rhizoidal growth of the bacterium *Bacillus mycoides* and several discrete colonies of other soil bacteria.

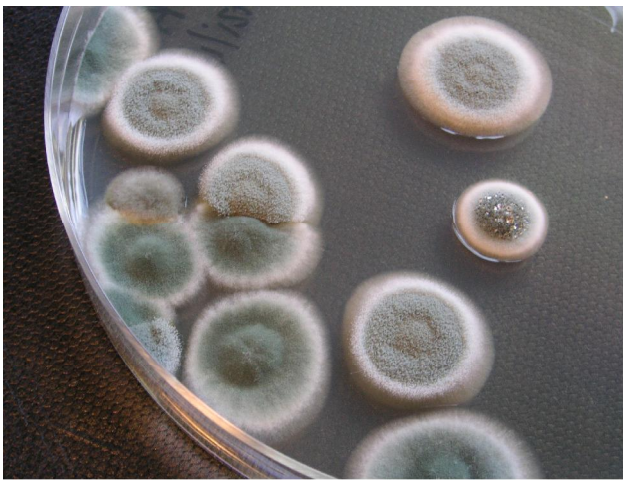
In the school context, little can be concluded about the identity of bacteria from solely the form of the colonies that develop on the surface of an agar culture medium. With one exception (vide infra), there is insufficient variety in appearances of their colonies (e.g. colour, size (up to a few millimetres), elevation, smooth or rough edge, shiny or dull surface) on a general-purpose culture medium such as nutrient agar to be of much help in their identification. Further information is needed from such classical procedures as microscopical examination of stained preparations and the study of a range of their biochemical properties. Techniques of molecular biology are increasingly being developed for use in rapid identification methods in research and routine diagnostic laboratories.

The exception referred to above is the unusual colony form of the bacterium *Bacillus mycoides* which commonly occurs in soil and dust, less frequently in air. To the naked eye the 'rhizoidal' growth that spreads for several centimetres over the agar surface superficially resembles the branched appearance of a fungus - or, more strictly, a mould ('moulds' and 'yeasts' are informal names given to two broad groups of fungi). However, the branched appearance is not a consequence of the individual cells being branched (which can be confirmed by microscopical examination) but by many chains of cells growing side-by-side across the agar surface and some changing direction to form a fork under the influence of variation in the gel structure of the medium.

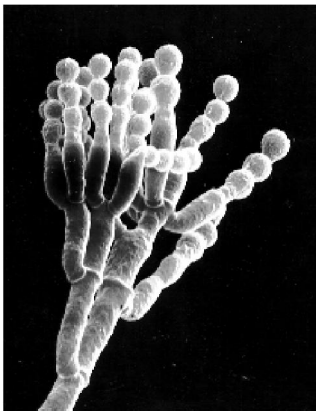
Professional microbiologists working in hospitals or the food industry are often sufficiently familiar with the range of bacteria they are likely to encounter to be able to draw conclusions about the identity of a culture from the appearance of its colonies. This is aided by using various special culture media that include ingredients designed to suppress the growth of bacteria that are not of interest but allow the development of pathogenic micro-organisms. MacConkey agar is an example of one such 'selective' medium.

NOTE 1: Colonies of yeasts are indistinguishable from those of bacteria to the naked eye and may also be present, especially if the culture medium contains added sugars, e.g. glucose nutrient agar, potato dextrose agar or malt extract agar.

NOTE 2: Colonies of moulds differ markedly in appearance from those of bacteria and yeasts. They are much larger, growing to a diameter of several centimetres and also are different in texture. Off-white, branched hyphae (the mycelium) spread over the surface of agar media. On continued incubation a dry, dusty, coloured (commonly white, green, black or orange) appearance develops on the surface of the growth as a result of spores being formed. Other than to the practised eye, such colour differences are of little value in identification for which it is necessary to make microscopical examinations of the hyphae and the shape and arrangement of the spores using low power and high power dry objective lenses.



Colonies of *Penicillium* sp. on malt extract agar medium after exposure to air.



Characteristic spore head of *Penicillium chrysogenum* showing chains of conidia (scanning electronmicro-graph).

We have had no success in showing zones of inhibition when testing for the effect of antibiotic discs on the growth of bacteria.

It may be that inoculated pour or spread/lawn plates of the test culture are being prepared a day or so ahead of when they are needed. This allows sufficient time for bacteria to start to grow at room temperature but not enough to be obvious. After the disc containing inhibitor has been placed in position, bacterial growth continues and ceases only when the concentration of the inhibitor diffusing from the disc reaches inhibitory levels. However, although growth has now ceased, that which has already occurred is still visible, thereby masking an expected zone of inhibition. Therefore, it is best to prepare the plates on the day and as near to the start of the practical class as possible. If this is impracticable the prepared plates could be refrigerated base uppermost for a short time, ideally no longer than overnight, but there may be condensation on the lid to contend with.

We are following an awarding body's suggestion to show inhibition zones using Yakult© for the test culture but no growth occurs.

The bacteria of Yakult© (and yoghurt), i.e. lactic acid bacteria, have exacting growth requirements that are not provided by the meat (and, sometimes, yeast) products that constitute nutrient agar medium. A fermentable substrate (glucose) and many other additional nutrients that they require are provided in MRS medium (named for the microbiologists de Mann, Rogosa and Sharpe who evolved it). The medium is available from biological suppliers.

A protocol for the isolation of *Rhizobium* from root nodules refers to mannitol yeast extract agar medium for culturing the bacteria but it is not listed in suppliers' catalogues. Is an alternative medium available?

Although mannitol is the carbon and energy source of preference, glucose is a suitable alternative; thus potato dextrose agar medium supplemented with 2.5 g/l yeast extract may be used*. However, if you are seeking a ready-prepared medium to which no further addition is necessary, glucose yeast extract agar medium is stocked by some suppliers.

HINT: It might also be of interest to observe *Rhizobium* bacteria directly in the nodules. Firmly crush a washed nodule in a loopful of tap water between two microscope slides. Treat the nodule squash as you would a smear and stain with crystal violet for 30 seconds. Careful examination with an oil immersion objective lens reveals Y- and club-shaped cells of *Rhizobium* among plant debris. These are known as 'bacteriods', the form of the bacteria within the nodule when they are fixing nitrogen. When free-living in soil or growing on the culture media described above, i.e. not fixing nitrogen, the cells are rod-shaped.

Root nodules on field beans (*Vicia faba*)

* See MiSAC's *Practical Microbiology for Secondary Schools*, pp 22-23, available free-of-charge from the publishers, the Society for General Microbiology (see Useful Links page for contact details).