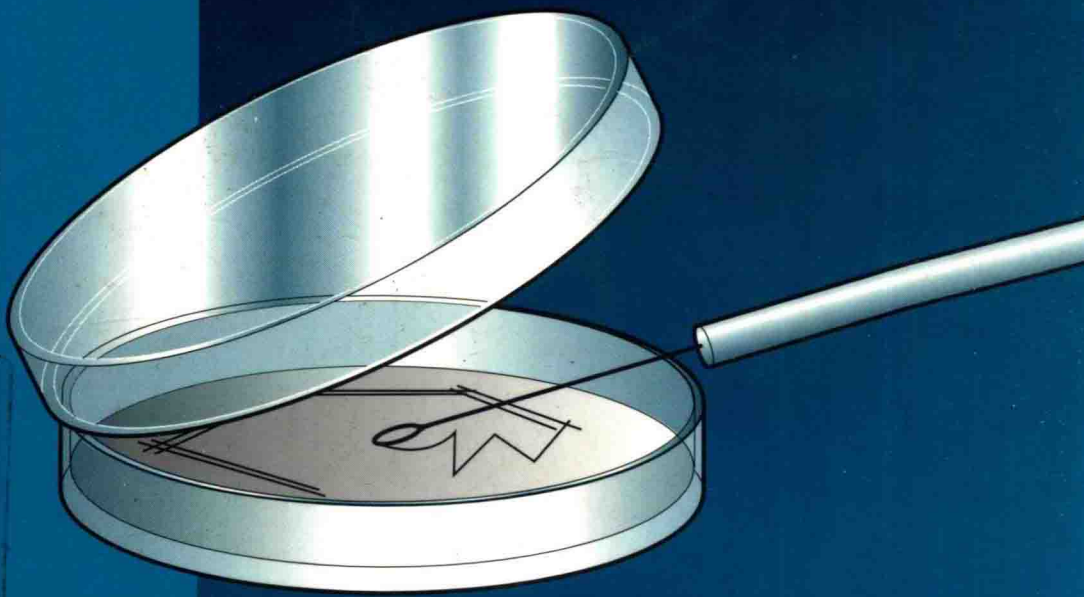
MICROBIAL CULTURE

Susan Isaac and David Jennings



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INTRODUCTION TO BIOTECHNIQUES

MICROBIAL CULTURE

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Abbreviations

A	adenine
ACDP	Advisory Committee on Dangerous Pathogens
C	cytosine
COSHH	Control of Substances Hazardous to Health
DAPI	4', 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
G	guanine
HPLC	high performance liquid chromatography
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
SEM	scanning electron microscopy
T	thymine
TEM	transmission electron microscopy
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume

Preface

Microbial Culture is intended to be an introductory text for those who are about to start growing micro-organisms. Such persons may be undergraduates, graduates, technicians or postdoctoral scientists. We anticipate that they are about to start their work in a laboratory in which some microbiological work is already being undertaken. So the emphasis here is on work at the bench with the necessary back-up of equipment and laboratory facilities. It could be said that if this is the case there should be no need for a book of this kind. But in our experience, the converse is true. In most microbiological laboratories, the knowledge possessed by a person joining is often assumed. Indeed, that knowledge may be tested not by prior questioning but by mistakes which can prove at a minimum inconvenient, but possibly very costly in terms of time, money and safety. In any case, passing on information just by word of mouth is never very efficient. On all counts, there is a need for a text to give persons new to growing micro-organisms the necessary background.

With the above in mind, we have written this book based on our own experiences, both good and bad! The great diversity of micro-organisms has meant that we have eschewed, for the most part, providing detailed protocols. We have taken the approach of highlighting matters to keep in mind when carrying out microbiological work. Effective protocols give every detail of the procedures to be followed. Circumstances, such as the organism used, the facilities available, etc., can all conspire to make it difficult to follow a protocol as written. Then there must be adaptation, and the success of such depends upon a proper understanding of what is involved. We hope this book will help with that understanding and provide a mental check-list of precautions and the like, which, if taken into account, will lead to the successful adaptation of any protocol to the specific needs of the investigator.

Throughout the text and with a supplementary list in Appendix C we have provided guidance about further reading. While that reading can provide the necessary assistance to address the particular problem of an investigator, we do recommend making contact with an expert in the field if it is proposed to start upon a field of microbiology

totally new to the investigator's laboratory. It is in similar spirit that occasionally we stress the need to make contact with the appropriate local expert over a particular issue, about which it is difficult to deal with all aspects within the somewhat limited confines of the present text.

Finally, we realize that often microalgae and protozoa are considered to be micro-organisms. We have made passing reference to these microbes but the bulk of the text concerns bacteria and fungi. This is partly due to our own experience, but also because we believe persons working with algae and protozoa are better served, because of the nature of the organisms, by referring to the relevant specialized texts on these organisms.

We are most grateful to our colleague Dr Alan McCarthy for reading the manuscript and giving us much helpful comment and advice. Chapter 6 has benefitted very considerably from the input of Dr Ron Burke who read the first draft. We thank him for his help, as indeed we are pleased to thank our editor, Dr John Graham, for his patience and constructive criticism.

Susan Isaac
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Contents

1 Introduction

1.1 Why culture microbes?

In order to be able to identify the kinds of microbes present in a sample, to estimate the profusion in which they are present and to investigate their particular metabolic properties one has to be able to cultivate them. In addition, by using laboratory cultures the number of variables, both chemical and physical, imposed on the organisms at any one time can be limited and controlled, allowing their responses to external factors to be assessed.

Cultivation aids accurate identification. It is necessary to grow up sufficient cells (biomass) of a species in order to be able to examine it and to establish its growth requirements. In the first instance, use of culture media which relate to the natural conditions in which the organism grows will usually allow cultivation, with subsequent experimentation leading to more precise definition of specific physiological requirements and genetic traits.

Microbes are extremely versatile and can show considerable morphological and/or physiological variation. They are to be found in almost every ecological niche, even seemingly hostile situations (such as hot springs), where it would be reasonable to assume that no organism could have the capacity to survive, let alone grow and reproduce. Additionally, many microbes readily form associations with other microbes, higher plants and animals as symbionts, parasites and pathogens. Such associations can range from those with mutually beneficial arrangements to those which have harmful or detrimental effects on one or both partners. Indeed, some microbes are not able to survive, grow and reproduce unless they remain in association with other organisms.

Large numbers (hundreds of thousands) of microbial species have been defined, each with its own particular characteristics and

requirements. More are being discovered and described, some with very unusual or unlikely life styles. It would be difficult therefore, if not impossible, to have a clear understanding of all microbial species and how each species is defined. Certainly, it is unnecessary to have such a detailed knowledge to be able to manipulate and successfully cultivate a wide range of micro-organisms. Here we provide guidelines to the principles and aims of microbial cultivation. In similar vein, in later chapters only pointers to more specialized methods are given, since these are obviously outside the scope of any single text.

Experimental work often has several aims. These might include the need to learn something about a very wide range of microbial species, inhabiting a particular ecological niche. Alternatively, very broad knowledge of a single species, or an in-depth study of one specific biochemical pathway operated by a species, may be required. Whatever the eventual aim or outcome of an investigation, it is clear that methods for growing individual species in *sterile* laboratory culture will be needed, and for this cells are usually placed into prepared growth medium. Such cultures are often termed *axenic,* meaning pure, uncontaminated cultures of a single organism. The researcher therefore requires an understanding of a range of techniques in order to become familiar with the organism(s) in which he or she is interested:

- pure culture for the isolation of single cells from the natural environment to an appropriate sterile growth medium;
- aseptic handling for successful transfers between different kinds of growth media;
- composition and preparation of growth media for different purposes;
- safe handling and storage of the isolated organisms in order to protect both the cultures and all higher organisms.

These topics will be covered in the first part of this volume, more specialized methods following in the second part.

As a group, micro-organisms have a very wide range of distinguishing characteristics, some of which can be determined simply and rapidly, while others require more time and specialized approaches:

- morphology of cells and colonies, for example shape, staining properties, motility, differentiated structures;
- growth characteristics, for example colony formation, nutrients needed, physical conditions;
- biochemical and molecular properties, for example secondary products formed, surface antigens, molecular characteristics (GC ratios, RNA sequences).

In general, microbes are very small and, as a result of a high surface area to volume ratio, are able to take up nutrients and lose toxic metabolites quickly. This often results in a very high metabolic rate. Generation times of 20–30 min have been recorded under conducive conditions. As a result, it is generally possible to obtain sufficient biomass of microbes in culture relatively quickly, which is a great aid to experimental investigations.

1.2 An introduction to the classification of micro-organisms

Most micro-organisms can only be seen properly with the aid of a microscope. However, many produce colonies that can be seen with the naked eye, and some form large, visually recognizable structures, for example mushrooms and algal fronds. There are huge numbers of different species of microbes, and classification groups together those with similar life styles and morphological and physiological characteristics. Some basic information about microbial classification is given here, indicating the major differences between the groups. The term micro-organism is usually used loosely, to cover (*Table 1.1*) viruses, bacteria (including actinomycetes and cyanobacteria), algae, fungi (including yeasts, lichens and slime molds) and protozoa.

TABLE 1.1: *A working classification of micro-organisms*

Organisms	Characteristics
Viruses	Noncellular organization
Prokaryotes	No nuclear membrane, no cell organelles
Eubacteria	Small, unicellular, often rods or cocci
actinomycetes	Filamentous organisms often found in soils
cyanobacteria	Photosynthetic prokaryotes
Archaebacteria	Small, unicellular
Eukaryotes	Nucleus delimited by membrane
Algae	Majority contain chlorophyll and photosynthesize like plants, others heterotrophic. May be unicellular and microscopic or multicellular and up to several meters in length
Fungi	Rigid cell walls, unicellular or filamentous and multicellular, frequently invasive of living and nonliving substrates. Heterotrophs, lack chlorophyll
yeasts	Unicellular, reproduce by fission or budding
slime molds	Lack rigid cell wall, form amoeboid aggregations
lichens	Symbiotic association between fungus and usually a single species of alga or cyanobacterium, resistant to harsh environmental conditions
Protozoa	Single celled, ingest particulate nutrients. Often flagellate, ciliate or amoeboid

The viruses fall uniquely into a group of their own since their organization is very different from that of cellular organisms. They are not capable of independent growth and are usually handled with particular specialized techniques. Viruses will not be considered further in this volume.

The ordering of species based on evolutionary relationships is known as phylogeny. Following the recent development of molecular sequencing techniques it has become possible to make comparisons between macromolecules in living organisms. This approach (molecular phylogeny) has shown that there are three cell types and has resulted in the definition of three kingdoms (now referred to as domains), each with very distinct evolutionary differences: Bacteria (formerly Eubacteria), Archaea (formerly Archaebacteria) and Eukarya (eukaryotes, including fungi, animals, plants and protista). Micro-organisms are also often divided on the basis of whether they are prokaryotes or eukaryotes. The distinguishing features are various (*Table 1.2*) but perhaps the most easily observed is that of size; prokaryotes are usually much smaller than eukaryotes. The arrangement of nuclear material is also a major feature. Prokaryotes (Bacteria and Archaea) do not have a nuclear membrane; the genetic material is carried in a single chromosome lying inside the cell. Eukaryotes (Eukarya) on the other hand, have nuclei; the genetic material is contained within a nuclear membrane in the cytoplasm and is packaged into a number of linear bodies or chromosomes. Other discrete structures (organelles), associated with particular biochemical activities, are also contained within the cytoplasm of eukaryote cells, accounting for their larger size.

1.2.1 Prokaryotes

Archaea (Archaebacteria). The cell walls of members of the Archaea consist of proteins or unique polysaccharides but those of bacteria contain peptidoglycan. The cell membranes also differ in structure. The membranes of archaebacteria are constructed from lipids bonded to glycerol by ether linkages, whereas in membranes of eubacteria straight chain fatty acids are ester linked to glycerol. There are also differences in the metabolism of the two divisions; some archaebacteria show highly specialized metabolic sequences, for example under anaerobic conditions some (methanogens) produce methane from carbon dioxide and hydrogen and a few can convert acetate to methane.

Archaebacteria are often found in unusual habitats. Some archaebacteria are extremely salt tolerant (extreme halophiles) and

TABLE 1.2: Major differences between prokaryotes and eukaryotes

Prokaryotes	Eukaryotes
Usually small	Larger than prokaryotes
Genetic material not surrounded by membrane	Nucleus membrane-bound
One chromosome	More than one linear chromosome
No meiosis	Mitosis and meiosis
No mitochondria	Most have mitochondria
Oxidative metabolism in cell membrane	Oxidative metabolism in mitochondria
No endoplasmic reticulum or Golgi apparatus	Endoplasmic reticulum and Golgi apparatus
Ribosomes (70S) dispersed in cytoplasm	Ribosomes (80S) attached to endoplasmic reticulum
No chloroplasts	Chloroplasts site of photosynthesis (if photosynthetic)
Photosynthesis in cell membrane (if photosynthetic)	Photosynthesis in chloroplasts
Peptidoglycans present in wall	No peptidoglycan
Flagella with one fibril	Flagella with 9+2 arrangement of microtubules and membrane

are found in salt lakes. They have a very high requirement for salt and are capable of growth only at near NaCl saturation. Others grow only at extremely high temperatures (100–110°C) and are dependent on elemental sulfur for growth. These are found in hot sulfur-rich springs, volcanic habitats and geothermal heated areas (vents) under the sea. Some (methanogens) produce methane under anoxic and anaerobic conditions. Methanogens are found in habitats rich in organic matter where oxygen levels are low, for example lake muds, swamps and the rumen of herbivores. Carbon dioxide, methyl-containing compounds or acetate are converted to methane by this group.

Bacteria (Eubacteria). The bacteria are subdivided primarily on the presence or absence of a cell wall. Those lacking walls are the mycoplasmas. The remaining bacteria are classified into groups (*Table 1.3*) based on the chemical composition of the cell wall (Gram stain; see Section 8.3.2), morphology, nutritional requirements and metabolic activity [1]. General accounts of the major groups of

TABLE 1.3: *Working classification of Bacteria and Archaea*

Group I Gram-negative bacteria with cell walls

Autotrophs
- Photoautotrophs (CO_2 fixed using light energy)
  - Oxygenic, photosynthetic bacteria (cyanobacteria), e.g. *Microcystis, Anabaena, Nostoc*
  - Anaerobic, anoxygenic photosynthetic bacteria, e.g. *Chromatium, Thiospirillum, Rhodobacter*
- Chemoautotrophs (CO_2 fixed by oxidation of inorganic compounds)
  - Ammonia and nitrite oxidizers, e.g. *Nitrosomonas, Nitrobacter*
  - Sulfides and sulfur oxidizers, e.g. *Thiobacillus, Beggiatoa*

Heterotrophs
- Aerobes/microaerophiles, rods and cocci, e.g. *Agrobacterium, Pseudomonas*
  - Pathogens of man and animals, e.g. *Brucella, Neisseria, Bordatella*
- Facultative anaerobes, motile or nonmotile rods, often in association with plants and animals,
  - *Enterobacteriaceae*-oxidase negative, e.g. *Erwinia, Escherichia, Salmonella*
  - *Vibrionaceae*-oxidase positive, e.g. *Aeromonas, Vibrio*
- Obligate anaerobes, isolated from intestinal tracts of man and animals, sewage sludge and anoxic muds, e.g. *Bacteroides, Veillionella*
  - Includes sulfate reducers, e.g. *Desulphobacter*

Miscellaneous
- Spirochaetes, e.g. *Leptospira, Treponema*
- Curved bacteria, e.g. *Campylobacter*
- Rickettsias and chlamydias (intracellular obligate parasites)
- Budding and appendaged bacteria, e.g. *Caulobacter*
- Sheathed bacteria, e.g. *Leptothrix*
- Gliding bacteria, e.g. *Cytophaga*
- Myxobacteria, fruiting bacteria, e.g. *Myxococcus*

Group II Gram-positive bacteria with cell walls

All are chemo-organotrophs (heterotrophs)

Endospore formers
- Form resistant endospores, often motile, aerobic or facultative aerobes, e.g. *Bacillus;* obligate anaerobes, e.g. *Clostridium*

Lactic acid bacteria
- Do not form spores, rods or cocci, catalase negative, microaerophiles, e.g. *Lactobacillus, Lactococcus, Streptococcus*

Actinomycetes and relatives
- Most form branching filaments, mostly aerobic or facultative anaerobes, e.g. *Mycobacterium, Streptomyces, Frankia*

Gram-positive cocci
- Chemo-organotrophs, do not form endospores, catalase positive,
  - facultative anaerobes, e.g. *Staphylococcus*
  - obligate aerobes, e.g. *Micrococcus*
  - obligate anaerobes, e.g. *Peptococcus*

Group III Bacteria lacking cell walls

Mycoplasmas
- Facultatively anaerobic to obligately anaerobic, some motile (gliding), e.g. *Mycoplasma, Spiroplasma*