HANDBOOK OF PRACTICAL BACTERIOLOGY

A Guide to Bacteriological Laboratory Work

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PREFACE TO THE REPRINT OF THE 9TH EDITION

PROFESSOR T. J. MACKIE was actively engaged in the revision of this reprint until his untimely death in October 1955. The staff in his department were closely associated with him in this work and have been mainly responsible for the additions in the Appendix of this reprint. They willingly undertook the completion of the revision.

I am grateful for their kind and ungrudging help as the distance between us is too great for close cooperation and easy communication. Without their assistance the Appendix could not have been completed.

The final edition of this revision has been carried out by Dr. R. H. A. Swain, Senior Lecturer in Bacteriology in the University of Edinburgh, with specialised contributions by Drs. Joyce D. Coghlan, J. P. Duguid, R. R. Gillies, F. L. Constable, J. C. Gould and H. A. Wright, also on the staff of the Bacteriology Department.

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PREFACE TO THE 9TH EDITION

THE publication of this ninth edition of the *Handbook of Practical Bacteriology* calls for little in the way of prefatory remarks. The text has been revised throughout on the basis of recent advances in bacteriological knowledge and technique, but the general character of the book has been continued from earlier editions and we have endeavoured also so far as possible to avoid any substantial expansion of the text by omitting methods (described in the previous edition) which have now been generally superseded by new procedures or have less application in bacteriological practice. We trust that the contents of the new edition will be acceptable and serviceable to students and laboratory workers, who may use it as a guide to the study and practice of bacteriology.

In the preparation of this edition we owe much to various colleagues who have given us advice and information on special topics, and we have to express our grateful thanks to the following for their generous help in this respect: Drs. J. H. Bowie, J. C. Broom, Joyce D. Cranfield, J. P. Duguid, W. M. Henderson, J. C. Gould, A. F. Maccabe, R. H. A. Swain, A. Wilson Taylor, A. T. Wallace, J. F. Wilkinson, D. R. Wilson, and Helen A. Wright.

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CHAPTER I

THE GENERAL BIOLOGY OF MICRO-ORGANISMS

THE BIOLOGICAL GROUPS REPRESENTED BY THE PATHOGENIC MICROBES

BACTERIOLOGY or Microbiology, as applied to medicine, embraces the study of those micro-organisms which are pathogenic to or commensals of man. The term "pathogenic" implies the power of producing disease; organisms which occur on the skin or in certain parts of the body—*e.g.* mouth, throat, intestine—without exerting any harmful effect are described as "commensals." Various commensal organisms are, however, potential pathogens, and some recognised pathogenic microbes may, under certain conditions, assume a commensal rôle, *e.g.* in the so-called infection carriers.

In veterinary science, bacteriology is specially concerned with the micro-organisms responsible for disease in domesticated animals. As many infective diseases are common to man and animals, medical and veterinary bacteriology are closely related branches of the general subject. Pathogenic organisms, however, show great diversity in their parasitism to different animal species, certain being associated with disease in the human species only, while others are highly virulent towards particular animals though non-pathogenic to man.

The pathogenic and commensal micro-organisms may be classified broadly in the following large biological groups: (*a*) BACTERIA or SCHIZOMYCETES ("fission fungi"); (*b*) FUNGI PROPER, which include the moulds and the yeasts; (*c*) PROTOZOA.

The exact biological relationships of some pathogenic micro-organisms, *e.g.* the Rickettsiae (which include the organism of typhus fever), still remain undetermined, and it is difficult to assign such forms to any one of these groups.

Moreover, certain infective agents are so minute that they can pass through filters which are impervious to the recognised bacteria and have been designated "filterable viruses" or simply "viruses." Some of these are beyond the range of

ordinary microscopic visibility and were at one time designated " ultramicroscopic." Certain viruses, however, have been demonstrated microscopically by special staining methods as minute bodies which are smaller than the bacteria, and many of them have now been observed as organised structures by electron microscopy. The nature of these viruses and the question whether they are organismal entities will be discussed in a later chapter.

The differential characterisation of the biological groups referred to is as follows :—

BACTERIA.—Organisms of microscopical dimensions and generally unicellular, though cellular units may be attached to one another and form chains, filaments or other aggregates ; morphologically simple as observed by ordinary microscopic methods ; multiply usually with great rapidity and by simple fission ; units spheroidal or cylindrical and rod-shaped, comma-shaped, spiral or filamentous ; devoid of chlorophyll ; do not exhibit a nucleus when unstained or stained by the ordinary methods, but nuclear bodies can be demonstrated by special methods ; certain species develop a resting-phase in the form of " spores " ; some of the filamentous types reproduce by means of " conidia " (p. 11) ; in certain species the cells are motile and possess flagella ; some forms are flexuous.

FUNGI : *Mould* forms.—Branching filaments (hyphae) interlacing and forming a meshwork (mycelium) ; more highly organised than the bacteria, often septate and multicellular, and reproduce usually by means of spores developed in " fruiting organs." *Yeast* forms.—Round, oval or elongated units, generally larger than bacteria and multiply by " budding " ; in certain species multiple " endospores " formed ; in some, hyphae occur.

PROTOZOA.—Generally regarded as the lowest forms of animal life ; minute unicellular organisms with the protoplasm well differentiated into nucleus and cytoplasm ; reproduce by fission and spore-formation, and often exhibit a definite life-cycle with both sexual and asexual phases.

The bacteria and the viruses play the most important part in the causation of human infective disease. Protozoal infections are most prevalent in tropical and sub-tropical countries.

THE BACTERIA

For practical work some scheme of biological classification is necessary. Various systems have been used, and one of

these, which has been elaborated in recent years, will be outlined later (p. 29). In the first instance, bacteria can be classified broadly as follows :—

HIGHER BACTERIA

Elongated and sometimes sheathed filaments, often showing true branching ; units may be interdependent, e.g. some being specialised for reproduction ; more highly organised than the lower bacteria—e.g. *Actinomyces* (p. 524).

LOWER BACTERIA (or EUBACTERIA)

Simple and typically unicellular structures, not in the form of sheathed filaments ; each unit biologically equivalent and autonomous ; many species motile, and this is usually associated with the possession of flagella.

Main Morphological Forms

1. Cocci—spheroidal in shape—*e.g.* the streptococci (p. 335).
2. Bacilli—relatively straight rod-shaped organisms—*e.g.* the typhoid bacillus (p. 434).
3. Vibrios and spirilla—definitely curved non-flexuous rods (vibrios) or spirals (spirilla)—e.g. *Vibrio cholerae* (p. 469).
4. Spirochaetes—flexuous spiral filaments—e.g. *Treponema pallidum* of syphilis (p. 538).

MORPHOLOGICAL STUDY OF THE BACTERIA

Unstained Preparations of Living Organisms.—The morphology of the bacteria can be studied in the first place by examining them microscopically in the unstained condition, suspended in fluid. In this way their general shape can be observed and motility determined (pp. 10, 70). Certain very slender bacteria, however, such as the spirochaetes, are so feebly refractile that they cannot be seen by the ordinary microscopic methods, and *dark-ground illumination* (p. 71) is necessary for their demonstration.

Electron microscopy (p. 81) is now applied to the morphological study of the bacteria, and has demonstrated many features not hitherto recognised.

For the study of the development of individual organisms and the growth of bacteria in communities or colonies (p. 14), the " agar-block " method of Ørskov, or the microscope-

incubator may be used (p. 227). These methods enable living bacteria to be observed at intervals during their actual growth on a suitable substrate, and present a more natural picture than other procedures involving manipulations which may sometimes create artificial appearances.

STAINED PREPARATIONS.—The microscopic examination of stained bacteria is usually an essential routine procedure. For this purpose various dyes, *e.g.* methylene blue, basic fuchsin, methyl violet, are employed, sometimes along with a mordant.

"*Negative*" *staining* is of value for the simple morphological study of bacteria—*i.e.* the organisms are mixed with some substance which, in film preparations, yields a dark or coloured background, while the organisms stand out as clear unstained objects. India ink and nigrosin are examples of substances used for this purpose (p. 88).

Silver impregnation methods (p. 113) are utilised for the staining of spirochaetes and are particularly applicable for demonstrating these organisms in tissues.

"Impression preparations" are valuable for the microscopic study of bacteria in their natural relationships in a colony on culture medium. For this purpose whole colonies are transferred to cover-slips and then suitably stained (p. 118).

STAINING REACTIONS.—The staining reactions of the bacteria are of the greatest importance both in their morphological study and for their differentiation and identification. Thus, all the bacteria can be divided into two categories by the so-called Gram's staining reaction (p. 89)—*i.e.* according to whether they resist decolorisation with aniline oil, alcohol or acetone after staining with a pararosaniline dye—*e.g.* crystal or methyl violet, and subsequent treatment with iodine. Those retaining the dye are designated "Gram-positive"; those decolorised by this method are spoken of as "Gram-negative."

The mechanism of the Gram-staining reaction is not yet fully understood. Gram-positive organisms are able to retain basic dyes at a higher hydrogen-ion concentration than the Gram-negative species, and it has been suggested that a greater affinity for a basic dye, due to a more acidic character of the protoplasm, may assist such retention. Another suggested explanation for the difference in Gram-reaction is that it depends on a difference in the permeability of the cell wall or the surface of the cell protoplasm. Thus, the dye-complex formed in the cell after staining and treatment with iodine can diffuse freely from the Gram-negative cell under the action of the decoloriser in

which it is soluble, but not from the Gram-positive organism, presumably because of a relative impermeability of its surface. Whatever the complete mechanism of the reaction may be, Gram-positivity appears to depend upon the integrity of the cellular structure and the presence in the cell of the magnesium salt of ribonucleic acid combined with protein; thus Gram-positive bacteria become Gram-negative if they are mechanically ruptured or if their magnesium ribonucleate is removed by treatment with bile salt, or separated, by means of the enzyme ribonuclease, from the protein with which it is combined at the surface of the cell protoplasm.

Some bacteria are relatively resistant to simple stains, but when stained by a strong staining solution (applied with heat) resist decolorisation by acid, and are spoken of as "acid-fast" —*e.g.* the tubercle bacillus (p. 397).

Such bacteria have a high lipoid content and their acid-fastness has been attributed to this. When lipoids are removed by extraction with suitable solvents, the cells are no longer acid-fast. One of the lipoids extracted from the tubercle bacillus, an unsaponifiable wax, known as "mycolic acid," exhibits the property of acid-fastness in the free state. But the mere presence in the cell of such lipoid is not in itself sufficient to explain acid-fastness, since the character is lost by autolysis or mechanical disintegration. Acid-fastness depends on the structural integrity of the cell and possibly on a special disposition of lipoid within the cell.

Certain bacteria do not stain uniformly. Thus, the diphtheria bacillus shows a "beaded" or "barred" appearance when stained with methylene blue. The plague bacillus displays "bipolar staining," the ends being more deeply coloured than the centre.

Such uneven staining may depend on various factors: nuclear and cytoplasmic constituents of the cell may stain with different intensity; the cell may contain granules with specially weak or strong affinity for the stain; and irregular appearances are sometimes the result of artifact.

Intracellular Granules.—In many species of bacteria, granules are observed in the protoplasm. These are not permanent or essential structures, and may be absent under certain conditions of growth. They appear to be lifeless aggregates of substances concerned in cell metabolism, *e.g.* reserve food material or waste products. They consist of fats, glycogen, starch, or "volutin," the last being possibly a metaphosphate, as indicated by studies of these granules in yeasts. The presence, nature and disposition of intracellular granules is characteristic of certain organisms and may aid in

their identification. Volutin granules are sometimes described as " metachromatic " on account of their peculiar staining reactions, having a strong affinity for basic dyes, and with these being more deeply coloured than the rest of the protoplasm. By special methods they may be stained with one dye while the rest of the cell is stained with another dye of different colour (p. 97). The diphtheria bacillus is one of the organisms exhibiting volutin granules and their demonstration is utilised for its identification.

Bacterial Protoplasm and Nuclear Bodies.—When unstained bacteria are examined, or bacteria stained by the usual methods, no differentiation into nucleus and cytoplasm is observed. However, by a special method it is possible to demonstrate the presence in bacteria of bodies which correspond to nuclei or chromosomes, though differing morphologically from the organised nuclei of animal or plant cells. After suitable fixation, the bacteria are treated with hydrochloric acid to reduce the affinity of the cytoplasm for stain ; on subsequent staining the nuclear bodies become deeply stained and the cytoplasm but slightly (p. 119). The nuclear bodies are oval or dumb-bell shaped and may be placed transversely in the cell. They react positively to the Feulgen test for desoxyribonucleic acid, an essential constituent of the nuclei of higher organisms. Unlike the intracellular granules described above, they are constantly present in all cells and under all conditions of culture. Only a single nuclear body is present in some cells, while in others, as a result of nuclear division preceding cell fission, two, four or even more nuclear bodies may be present.

Cell Wall and Cytoplasmic Membrane.—The bacterial substance is contained within a very thin *cytoplasmic membrane*, and external to this a relatively rigid *cell wall*. It is this latter structure which maintains the characteristic shape of the organism. The cell wall appears to be composed of protein combined with lipoid or polysaccharide. By the usual staining methods it remains invisible, but may be revealed by special methods : by staining after treatment with tannic acid, or after treatment with strong salt or alkali solution to cause shrinkage of the protoplasm. The cell wall is clearly demonstrated by electron microscopy.

Bacterial Capsules and Extracellular Slime.—Certain bacteria may exhibit a relatively thick outer capsule and are described as " capsulate." The capsule is a structure quite

distinct from the cell wall and lies outside it. The development of capsules is usually dependent on certain favourable environmental conditions, *e.g.* the presence of abundant carbohydrate and, in the case of pathogenic bacteria, growth in animal tissues or in a culture medium enriched with unaltered animal protein. In certain species complex carbohydrate substances of polysaccharide nature enter into the composition of such capsules, in other species polypeptides or proteins; such substances are of great importance in determining specific characters (p. 39). Virulence may also depend on capsule formation (*vide* pneumococcus, p. 356). By ordinary methods of staining the capsule is invisible, though in microscopic preparations from animal tissues or body fluids it often appears as an unstained zone round the organism. Special methods are available for the differential staining of capsules (p. 100). "Negative" staining is also of particular value for demonstrating capsules (p. 88).

Many capsulate organisms and some non-capsulate species produce extracellular slime. This is a colloidal viscid material which is similar in chemical nature to capsular polysaccharides. The slime is situated outside the cells and their capsules. It is amorphous and does not constitute a definite structure like a capsule; it also readily disperses into solution in liquid culture medium, though in growths on solid medium it remains as a matrix in which the bacteria are embedded, conferring on the growths a viscid or "mucoid" character. Slime can be demonstrated by some of the methods applicable to capsules, *e.g.* the India ink method (p. 88).

Bacterial Spores.—Some species, mainly those of bacillary morphology, develop a highly resistant resting-phase or spore, by which the individual can survive unfavourable external conditions. The spore is not a reproductive structure. In the vast majority of spore-bearing species only one spore is developed by each vegetative cell. The spore is formed in the protoplasm (hence called "endospore") and includes part of the nuclear material of the cell. As it develops it acquires a dense outer envelope ("spore case" or "spore coat") and becomes resistant to ordinary staining. When mature it takes up a "central," "subterminal" or "terminal" position. The relative size of the spore varies with different species. It may be spherical, oval or elongated. Spores can withstand all injurious chemical and physical influences better than the vegetative forms, and probably owe their resistant properties to their envelopes and their low

content of unbound water, though the total water-content of spores and vegetative forms may be the same. Spores also differ from vegetative cells in lacking enzymic activity. They may be antigenically different from the vegetative phase (p. 38), which indicates some essential difference in chemical constitution.

Under favourable external conditions, *e.g.* as regards the presence of moisture and nutrient materials, the envelope ruptures at one pole or equatorially, and the vegetative organism then emerges. This process is described as the " germination " of the spore. Sometimes the envelope disappears without obvious rupture. By electron microscopy it has been shown that this envelope is a structure quite distinct from the vegetative form which emerges from it.

In unstained preparations the spore is a highly refractile body as compared with the vegetative cell. It is not stained by the ordinary methods but appears as a clear, unstained portion of the bacterial structure. In some cases, however, the spore stains by Gram's method. Special methods are employed for the differential staining of spores (p. 99).

MOTILITY.—The motility of bacteria (observed in a fluid medium) has generally been regarded as due to delicate prolongations of the protoplasm (flagella) which act as locomotory organs; and their propellent action has been described as the result of contractions moving spirally over their surface. They are not seen in unstained preparations and can be demonstrated only by special staining methods (p. 102) or by electron microscopy. Brownian movement must not be confused with true motility. In the latter case the organism definitely changes its position in the microscope field (p. 71). Brownian movement is due to the impact of the molecules of the medium in which the organisms are suspended (" molecular bombardment "), and is an oscillatory movement within a limited radius. (See **Appendix**, Chap. VI.)

Flagella may be " terminal " (or " polar ")—*i.e.* at one or both ends of the bacterium—and single or multiple. They may appear in stained preparations to be distributed all round the organism, and this arrangement is described as " peritrichous "; but possibly such disposition may not be a natural one when the organism is in motion. Thus, under such conditions the flagella may be coiled into a single spiral " tail " which projects from one end.

It has been claimed by Pijper that the motive force of various bacteria resides in the protoplasm and is due to wave-like spiral con-

tractions which are communicated to the cell wall. He suggests that the flagella are the products of movement and derived in this way from mucoid material on the surface of the cell. Electron microscopy, however, has clearly shown that flagella are organised structures as originally supposed, and originate from the cytoplasm.

When there is a single terminal flagellum the term " monotrichous " can be applied ; " amphitrichous " indicates the presence of a flagellum at each pole ; " lophotrichous " refers to the arrangement of multiple flagella at one or both poles.

Among the spirochaetes motility is generally considered to be a function of the contractility of the cell protoplasm. However, *filamentous* flagella-like structures, have been demonstrated in certain spirochaetes, e.g. *Treponema pallidum*, by staining and electron microscopical methods. (See **Appendix.**) The most characteristic movement is a spiral rotation on the long axis with progression in the axial line. Movements of flexion, and sometimes lashing movements, are also observed.

Pleomorphism and Involution.—It must be remembered that, especially in artificial culture, bacteria may show considerable variation in shape and size (pleomorphism), and may also exhibit degeneration or " involution " forms which are different morphologically from the normal cell. Such abnormal forms are commonly found when bacteria are growing in the presence of antagonistic substances, or when cultures on artificial media are ageing.

Multiplication among the Bacteria.—Among the lower bacteria, multiplication takes place by simple binary fission. The cell enlarges or elongates, and the protoplasm becomes divided transversely or equatorially. In some species, division of the cell wall immediately follows, and two free and independent units are formed ; in others, the cell walls of the new individuals remain continuous (as is well demonstrated by electron microscopy) and the organisms tend to adhere and grow in pairs, chains or clusters (*vide infra*). Division may occur with great rapidity, *e.g* every half-hour, so that one individual may soon reproduce several millions of new organisms. Among the spirochaetes transverse fission occurs as in other bacteria.

In the higher bacteria transverse division of the filaments into shorter forms is observed. Certain filaments also form at their free ends a chain of oval " conidia," which are set free, and each of these, besides representing a resting-phase, may develop a new colony.