THE CYTOLOGY AND LIFE-HISTORY OF BACTERIA

BY

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Reader in Systematic Bacteriology
University of Birmingham

Second Edition

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Preface to Second Edition

THE second edition of this monograph includes a considerable amount of new information which serves, for the most part, to confirm and expand the general theories of cytological structure and behaviour in bacteria which I advanced six years ago. In particular, greatly improved demonstrations have been achieved of such, formerly rather problematical processes as gonidial reproduction, nuclear reduction, and the development of the flagella, and of such structures as the blepharoplast or the startlingly complex cross-walls which sub-divide a staphylococcus internally.

I have continued to place the main weight of my arguments upon my own observations or upon information which I have been able personally to confirm, but have welcomed several remarkable contributions to knowledge in the form, for example, of Chapman and Hillier's electron micrographs of sections of *Bacillus cereus*, which prove, contrary to my previous belief, that the cross-wall is indeed a centripetal ingrowth, and the consummately skilful phase contrast studies of Tomcsik which, while demonstrating the profundity of my former ignorance of the nature of bacterial capsules, provide a gratifying confirmation of my hypothesis, based upon entirely different evidence, of the development of cell wall.

Since the first edition was published there has also been a notable increase in the amount of corroborative evidence provided by studies in genetics, biochemistry and biophysics. The single, reductionally dividing chromosome of the vegetative nucleus, which has been the subject of the most lucid cytological demonstrations, has been entirely vindicated by the genetical studies of Witkin and of Cavalli-Sforza and Jinks, after a period in which multiple, or even branched chromosomes, for the existence of which there is no acceptable cytological evidence, were at various times postulated by geneticists. And in a similar manner, the cytological and physico-chemical evidence upon the behaviour of cell envelopes and flagella have been in exceedingly close accordance, and have even begun to shed some light upon the problems of antigenic structure.

Since it is now the subject of a separate monograph (*Bacteria*, Livingstone), the chapter upon bacterial systematics has been omitted, but the allied problem, more cogent to this study, of cytological evidence of the evolutionary relationships of bacteria, has been discussed at greater length.

Because the text is intended to be a synthesis of available information, the practice of relegating references to the head of each sub-section, except in cases of argument or of historical interest, has been continued. There appears to be a consensus of agreement that what may be lost in ease of tracing a reference to a single point is more than gained in clarity and brevity.

April 1955 K. A. BISSET.

Preface to First Edition

THIS book does not attempt to review the literature upon bacterial cytology, of which the bulk is very great and the value, in many cases, difficult to assess. The bibliography is confined to a relatively small number of works, almost all recent. No attempt has been made to supply references for analytical discussion or general information.

The purpose is rather to present a reasoned case for regarding bacteria as living cells with the same structure and functions as other living cells, and to correlate the available information upon the various types of bacteria.

Bacteria, as living creatures, have been little studied. It is their activities as biochemical or pathological agents which have received almost undivided attention. Even these problems, however, cannot fail to be clarified by a better knowledge of the organisms responsible.

It is also hoped that biological workers in other fields may profit by contact with this, largely unknown, body of evidence, and may find the comparisons and analogies useful and stimulating in their related studies.

I have attempted, as far as possible, to base my arguments upon my own observations, or upon such information as I have been able personally to confirm. Where I have not had the opportunity to do so, I have tried to indicate clearly the status of the argument.

K. A. B.

December 1949

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CHAPTER I

Introduction

MUCH of what has in the past been written of the morphology of bacteria has been based upon the assumption that, because of their small size, and the difficulty, by the methods usually employed, of observing the complexities of their structure, they may be regarded as simple in form and primitive in philogeny.

The temptation to regard small size, and simplicity of structure, whether real or apparent, as criteria of a primitive condition, has often proved the cause of error and confusion in the classification of other groups of living organisms. As more information becomes available it is almost invariably discovered that the simplest creatures exhibit characters which suggest a relationship with others, much more complex, or may themselves prove to be less simple than they had been believed. This has proved to be true of bacteria also. Although for long believed, in spite of much evidence to the contrary, to be almost structureless cells, reproducing by simple fission, they have proved to possess an intricacy of structure rivalling that of any other type of living cell, and to undergo life-cycles of considerable complexity.

There is little doubt that the reason why so much more has been learned of the physiology of bacteria than of their morphology, is their very great importance in medicine, industry and agriculture. The immediate, practical problems of bacteriology have overshadowed the more academic questions of their biological nature. The techniques which were devised for the solution of these problems have been notable, in almost every case, for their failure to provide even a minimum of basic, biological information. Indeed it may be said that much of the information of this nature, accumulated since the commencement of systematic bacteriology, has tended rather to obscure than to clarify the underlying truths.

Especially is this true of the staining techniques employed for routine

examination of bacteriological material and cultures. The distorted vestiges of bacteria which survived the technique of drying and heat-fixation were accepted as truly indicative of the morphology of the living organisms. And while, from time to time, satisfaction has been expressed at the fact that bacteria will survive, undistorted, treatment which produces the most obvious damage in larger cells, the validity of the assumption that they do, in fact, survive such treatment has seldom been called to question.

Staining methods have also been devised, almost without exception, for the purpose of identifying clinically important species of bacteria, and are often most admirably suited to this task. It is surprising to find, however, that much time and labour has been directed to the elucidation of the appearances observed by these methods, and the explanation, in cytological terms, of the artefacts which they produce.

Even with this disability a great deal of accurate information has in fact been obtained, but has failed to carry conviction. In many cases this has been because of inadequate illustration, which alone can make such studies comprehensible, except to the initiate. Probably the reason has been an unduly pessimistic view of the possibilities of photomicrography, and a certain timidity in the submission of drawings and diagrams, due perhaps to a fear of misinterpreting such tiny structures, and a corresponding fear of ridicule.

It is also remarkable that many workers in the field of bacterial cytology appear to have been almost entirely ignorant of the parallel studies of others, and have failed to receive the stimulus which such knowledge can afford. Conversely, there has been no lack of reviews of the subject, but these have often been made by authors whose lack of practical knowledge of the structures described has disqualified them for the task of correlating the available information, which is often obscure and mutually contradictory.

The artificiality of contemporary or recent views upon bacterial morphology has thus served to widen the gap between bacteriology and other biological sciences, as well as to confuse and retard the advance of bacteriology itself.

In the evolution of modern cytological methods, much is owed to the interest taken by mycologists in the myxobacteria. These micro-organisms do not respond well to the techniques of heat-fixation and Gram's stain, most

usually employed in routine bacteriology, and the necessity for the employment of more refined methods of examination has encouraged the study of eubacteria in a similar manner. The readily-demonstrable nuclear structures and beautiful and complex life-cycle of myxobacteria stimulated the search for the truth concerning the parallel structures and processes in those bacterial genera more commonly encountered in the laboratory.

The studies of biochemists upon the nucleoproteins of bacteria have also contributed greatly to the increase in our knowledge of, and interest in, the problems of bacterial cytology. One of the most useful staining techniques for the demonstration of the bacterial nucleus is a direct adaptation of a microchemical test, the Feulgen reaction, which has itself given much information upon the subject.

Bacteria have recently come to be regarded as suitable material for genetical studies, and although little has so far been done to correlate genetical and cytological information, a gratifying degree of mutual support has already been achieved (Chapter X), and it is to be hoped that the interchange of information between these two branches of bacteriology may, in the future, prove as helpful to both as it has done in other biological fields.

The information compiled in the following chapters has been obtained by classical microscopic methods, in most instances, but a considerable advance in the techniques of electron and phase-contrast microscopy, as applied to this subject, has in the last few years provided valuable confirmatory evidence on several points, and promises to do more. It should be emphasised that a reasonable degree of correlation between the results obtainable by different techniques must always be sought before too much weight is placed upon any one of these. The disagreements which have arisen in bacterial cytology have been surprisingly few. But almost all of these have been caused by the uncritical reliance of a single worker, or a small group, upon a single method.

CHAPTER II

Technique

A: THE STAINING OF BACTERIA

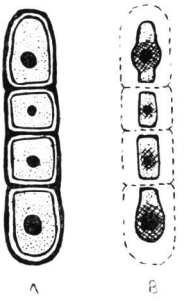
(8, 11, 53)

THE progress of our knowledge of bacterial morphology has, in the past, been considerably retarded by the fact, which may at first have appeared advantageous, that recognisable microscopic preparations of bacteria can be made by the technique of the heat-fixed film. A small quantity of a bacterial culture, or of pus, or similar pathological material, is thinly spread upon a slide, dried, and then heated strongly with a naked flame, in order to fix it firmly upon the slide. Bacteria fixed in this manner and stained by Gram's method, or simply with a strong solution of a basic dye, dried once more and examined directly under the oil-immersion lens of the microscope (the oil serving also as a clearing agent), preserve an appearance which enables them to be recognised as bacteria, and even classified within rather broad limits. Their appearance under this treatment has become familiar to generations of bacteriologists, and is usually that which is recorded in the descriptions of species. Little or no detail can be perceived in such a preparation, and it has thus become, and until recently has remained a dogma that no detail exists to be seen. This opinion is fortified by the fact that equally little structure can, as a rule, be made out in unstained, living bacteria, especially as these are seldom at rest, either because of their own motility or from the effect of Brownian movement.

It is true that, from time to time, valuable observations upon the structure of bacteria have been made, either by the cytological techniques already employed in other biological sciences, or by a careful study of unstained material, but little attention has been paid to these findings by the great

majority of bacteriologists, and the interpretation of heat-fixed material has not been questioned seriously.

The main reason for the uniform appearance of stained bacteria is that their affinity for the basic dyes which are commonly employed is so great that the strongly stained cytoplasm and cell membranes mask the underlying structures. This masking effect is accentuated by the shrinkage which results from drying. This shrinkage is often very considerable, reducing the bacterium to as little as half or a third of its natural size, and manifesting itself typically in the appearance of the anthrax bacillus or of related chain-forming bacilli,



(Reproduced from the Journal of General Microbiology)

FIG. 1.

THE MORPHOLOGY OF *C. DIPHTHERIAE*.

A. True morphology.

B. "Typical appearance" in heat-fixed material. The cell contents are shrunken and the cell wall unstained.

in which considerable gaps are seen between the visible bacilli, actually the shrunken protoplasts. The rigid cell wall remains unstained and invisible, holding the chain together. Drying and shrinkage are an essential part of many staining procedures, notably those intended to demonstrate the "typical morphology" of *Corynebacterium diphtheriæ*. The metachromatic granules cannot be demonstrated in undried preparations, and are, in fact, artefacts produced by the specific staining of a dried aggregate of nuclear and other basophilic material.

Many bacteria are multicellular, and their appearance is much altered by drying. The granular appearance of the tubercle bacillus is due to the shrinkage of the contents of the small, almost spherical cells which make up the bacillus, so that unstained gaps appear between them. In this case also the cell wall remains unstained, but retains the dried cells in their original relationship.

It will thus be seen that three main problems must be solved in the demonstration of the true morphology of bacteria. Distortion due to drying must be avoided, the masking effect of the strongly staining protoplasm and cell membranes overcome, and those structures demonstrated which, like the cell wall, are difficult to stain. The first is simple and entails merely the avoidance of drying at all stages of preparation. The second and third present more difficulty. The problem of overcoming the masking effect of the surface structures was solved, as so often happens, by accident.

B: HYDROLYSIS TECHNIQUES FOR NUCLEAR STAINING

(13, 15, 16, 19, 24, 30, 32, 40, 41, 44, 46, 48, 50, 51, 52, 53, 57)

The Feulgen reaction is a microchemical test which depends upon the formation of a purple compound when aldehydes react with Schiff's reagent. A positive Feulgen reaction is given by deoxyribose nucleic acid, after its purine bases have been removed by acid hydrolysis. Ribonucleic acid does not give a positive reaction. The hydrolysis is performed in Normal hydrochloric acid at a temperature of 60° C., and the subsequent staining with Schiff's reagent reveals the nuclear structures of bacteria with reasonable clarity. This was one of the first methods to give a true picture of the bacterial nucleus, and it was later discovered that if the final staining was performed with Giemsa's solution, instead of Schiff's reagent, a much clearer picture was obtained. This was the acid-Giemsa stain, which has been the basis of nearly all recent work upon the bacterial nucleus, although the information which it provides can be verified by other methods.

The purpose of the preliminary treatment with hydrochloric acid is two-fold. The nucleoproteins of the underlying structures are partially hydrolysed so that the aldehyde group of the associated pentose sugar is

released and combines with the staining agent. At the same time the stainable material of the outer layers of the cell is more completely hydrolysed, so that its masking effect is reduced. This differentiation is made possible by the fact that the nuclear structures are composed largely of Feulgen-positive deoxyribose nucleoproteins, whereas the cell membrane and surface layers of the cytoplasm usually contain a higher proportion of ribose nucleoproteins.

To perform the stain, smear preparations are made upon slides or coverslips. They may be unfixed, although these tend to wash off, or they may be fixed in osmic acid vapour. Most fixatives should be avoided as they may completely alter the appearance of the nucleus.

Hydrolysis in Normal HCl should be conducted at a temperature, approximately, of 60° C. Staining, in dilute Giemsa, is best performed at 37° C.

The periods required for hydrolysis and staining are exceedingly variable and may be different at different ages of the same culture. It is often necessary to examine the preparation with the microscope, in order to determine whether it is suitably stained, and for this purpose a water-immersion lens is a great convenience. Most bacteria require from ten to twenty minutes hydrolysis, and thirty minutes in the staining solution. Some require longer periods or stronger solutions.

A properly stained preparation is bright pink in colour, the nuclear structures staining more intensely than the cytoplasm, which may stain bluish or purple in some cases. Inadequate hydrolysis is indicated by a uniform purple colour, and excessive hydrolysis by a pale pink colour and blurred outline. Inadequate or excessive staining periods are self-evident in the appearance of the preparation.

It is important to use fresh reagents, and otherwise inexplicable failures may be found to be due to neglect to do so.

Other methods of staining give comparable results, and may be useful in the case of bacteria which do not stain well by the classical method. Cold perchloric or trichloracetic acid, or even weak alkalis, may be substituted for hydrochloric acid. A variety of different dyes may be used instead of Giemsa. Thionin gives good results and has been widely used, but is much less specific than Giemsa, and stains the basophilic elements of the cell envelopes as well as the nucleus, which is liable to cause confusion in interpretation.

C: DIFFERENTIATION TECHNIQUES

(2, 10, 13, 20, 48, 66)

The methylene-blue-eosin method has been used to demonstrate the nuclear material of bacteria which will not stain readily by acid-Giemsa, it is unfortunately irregular in its results and may be liable to produce artefacts.

Basically the method is exceedingly simple. The preparation is stained with aqueous methylene blue and differentiated with eosin. The cytoplasm stains pale blue, and the nuclear structures dark blue or purple. In practice, however, it is a difficult technique to perform, and is not suitable for all strains of bacteria.

The film should be made thick and stained until dark blue throughout. It is then washed in water, differentiated for a few seconds in eosin and immediately washed again. The action of the eosin is very rapid, and it will entirely remove the blue colour if it is allowed to act for too long.

It was noted previously that this technique may usefully be employed upon bacteria which resist staining by acid-Giemsa, and the converse is also true. For this reason, methylene-blue-eosin is best regarded as a useful adjunct to acid-Giemsa, and is not recommended as a routine cytological method.

Similar results are obtainable by the use of crystal violet, with nigrosin as a differentiating agent.

D: THE ROMANOWSKY STAINS

(36, 47)

The methylene-blue-eosin technique differs from the better-known staining methods of the Romanowsky type in that the combination of the acidic and basic dyes is permitted to take place during the period of the staining reaction. The more orthodox methods are often of considerable value, however, and simple staining with Giemsa will often prove of value in the case of bacteria, such as myxobacteria and some members of other orders, whose surface structures lack the strong affinity for dyes exhibited by many. Valuable observations have been made in a variety of bacterial groups by the use of these methods.

E: SIMPLE DYES

(1, 14, 58, 59)

Even the simple dyes, especially basic fuchsin and methylene blue, may be of value upon occasion, if the errors of heat-fixation and drying, which usually accompany their use, can be avoided. The affinity of bacterial cytoplasm for the basic dyes is so great that a short treatment will often produce an appearance of negative staining of the nuclear structures, which appear pale and refractive against the stained background. This phenomenon is well known, and is usually described as bipolar staining. Accumulations of basophilic material at the poles are also associated with the growth of the cell. Reagents and even displaced nucleic acids may form aggregates in these areas and appear as granular artefacts.

An interesting refinement in the use of a simple dye, which has been employed with considerable success, consists in permitting a thin film of carbol fuchsin to dry upon a slide. The bacteria are suspended in a drop of water upon the coverslip which is inverted upon the slide and sealed at the edges. The dye is taken up gradually by the bacteria and the process may be followed under the microscope.

This method has proved of value in the description of certain of the complex processes which precede the formation of the resting nucleus, but appears to have failed to demonstrate the active, vegetative condition of the nucleus in the same species of bacteria.

F: THE USE OF PROTEIN MATERIALS

(17, 25, 60, 61, 62, 63, 64, 65, 68)

As the surface material, the affinity of which for basic dyes tends to obscure the internal structures of bacteria, is composed mainly of ribose nucleic acid, it has been found possible to digest away this material with the enzyme ribonuclease. This leaves unharmed the deoxyribose nucleic acids of the nucleus itself, which can then be demonstrated without difficulty.