METHODS IN MICROBIOLOGY

VOLUME 6A

METHODS in MICROBIOLOGY

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PREFACE

The main theme in Volume 6 of "Methods in Microbiology" is the application of biochemical techniques to the study of micro-organisms. The topics covered include the use of biochemical and enzymic tests to characterize microbial types, the quantitative separation and analysis of fermentation products produced by micro-organisms, a critical appraisal of methods available to elucidate metabolic pathways and the control of enzyme synthesis, the assay of selected enzymes in crude extracts of cells as indicators of metabolic pathways, the use of antimetabolites to study biosynthesis and electron transport. The applications of radiotracer techniques are described in detail in five chapters: respiratory measurements with dyes and with electrodes for oxygen and carbon dioxide are included while manometric methods, which are so well described elsewhere (Umbreit, Burris and Stauffer, 1964) have been excluded from this series; articles on nitrogen fixation, calorimetry, electrochemical measurements and methods for studying sporulation and germination are also provided.

The detailed choice of the contents of the various contributions has been left largely to the individual authors. We have, as in the past, edited only to conserve consistency, bridge the gaps and avoid, where possible, overlaps between the articles.

Volume 6, like Volumes 3 and 5, has been divided into two parts. The division of the contributions, although somewhat arbitrary, was made by grouping the more chemical and enzymological topics into Volume 6A and allocating the more physical techniques of isotopes, electrodes, electrometry and calorimetry to Volume 6B.

We are grateful for the pleasant way in which our contributors have co-operated with us during the last three or four years. We must particularly thank those authors who have had the patience to wait for this publication. Some completed manuscripts were received three years ago, and many were subsequently revised by their authors.

J. R. NORRIS

D. W. RIBBONS

September, 1971

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CHAPTER I

Routine Biochemical Tests

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I. GENERAL PRINCIPLES

The selection of a reasonable range of biochemical tests to be performed as a routine for bacterial identification and characterization is dependent upon the source of the material, the diversity of the bacteria likely to be encountered and the aim of the investigation. In general, details of less common tests applicable to special groups of bacteria are listed elsewhere in these volumes, but special tests in common use are also included in this chapter.

Before initiating any detailed examination of a bacterial isolate, the purity of the culture must be confirmed; this is particularly important if colonies have been picked from selective media. Serial subculture on a non-selective medium may be necessary to remove persistent contaminating organisms. The Gram staining procedure, which is usually done at an early stage in the investigation, allows observations on size, shape, and arrangement of cells, and gives provisional information on spore formation. Microscopic examination of a wet preparation from a broth culture may yield information on motility. However, morphology, staining reactions and motility are affected by various factors such as the age of the culture and the composition of the medium.

The reproducibility of a biochemical test is of fundamental importance if the test is to be a reliable step in an identification procedure. Ideally, all the details of test culture media and growth conditions should be defined and strictly observed. The absolute responses sought in classifying bacteria are frequently contrived by adjusting methods and it is increasingly important that methods should be internationally standardized and performed with care. Controls including known positive and negative tests should always be included with routine tests.

Frequently, the only quantitative measurements recorded are the time and temperature at which changes occur. Little attention appears to be paid to such important points as the size of the inoculum, the volume of the test medium, or the type of container to be used. In many tests a standard inoculum pipetted from a liquid culture or a homogeneous suspension of

the test organism will prove more reliable than a loopful of growth from an agar slope or plate culture. In general, a small inoculum, e.g. a drop of a slightly turbid homogeneous suspension of an actively growing culture of the organism in saline, is recommended. The stage of growth of the test inoculum may influence the result. In tests that depend upon the detection of end-products of bacterial decomposition of a substrate incorporated in the test culture medium, the ability of the organism to grow in the test medium without the added substrate should normally first be confirmed before a negative result is recorded. Development of turbidity is usually accepted as evidence of growth, but some media develop floccular precipitates after autoclaving or during incubation and this may be misleading. The aseptic addition of separately sterilized salts can often avoid this complication. If precipitation is suspected after incubation, it is necessary to confirm microscopically that growth has occurred.

The development of acidity or alkalinity is frequently demonstrated by a colour change produced by a pH indicator incorporated in the test medium. The disadvantages of the use of pH indicators in this way merit consideration:

(i) Bacterial growth produces changes in redox potential as well as changes in pH. Many indicators are bleached or rendered colourless under the relatively anaerobic conditions that can develop in an actively growing culture. In some cases the change is not readily reversible and it may misleadingly suggest that a pH change has occurred.

(ii) Some indicator solutions are antibacterial and may inhibit or diminish growth.

(iii) Indicator solutions should not be incorporated in culture media that are already strongly coloured by the ingredients, or in cultures that may develop colour as a result of bacterial pigment production.

It therefore follows that pH indicators should be carefully chosen. There is often much to commend the practice of growing the test organism in broth containing the test substrate and of spot-testing the broth culture on a porcelain tile with an indicator that is not incorporated in the medium.

Many media can be sterilized in the autoclave by exposure to wet heat at 121°C (pure steam at 15 lb/in^2 above normal atmospheric pressure) for 15 min. Except where otherwise stated in the chapter, this procedure is recommended.

II. THE DECOMPOSITION OF SIMPLE CARBOHYDRATES, ORGANIC ACIDS AND SOME OTHER COMPOUNDS

The biochemical tests discussed in this section demonstrate one or more of the following:

(a) the ability of the organism to utilize the test compound as a sole source of carbon and energy;

(b) whether oxygen is required for utilization to take place, i.e. whether the organism is oxidizing or fermenting the substrate or is capable of carrying out both processes; and

(c) the detection of an end-product of the metabolism of a compound that is not necessarily serving as an energy source.

A. Demonstration of a sole source of carbon and energy for growth

Large numbers of compounds can readily be tested by using methods similar to those described by Stanier, Palleroni, and Doudoroff (1966) for aerobic pseudomonads. The mineral basal medium contains per litre:

($Na_2HPO_4 + KH_2PO_4$) buffer (1M; pH 6·8)	40 ml
$(NH_4)_2SO_4$	1·0 g
Hutner's vitamin-free mineral salts solution	20 ml

The Hutner mineral salts solution (see Cohen-Bazire *et al.*, 1957) which can be replaced by alternative solutions contains the following ingredients per litre:

nitrilotriacetic acid	10 g
$MgSO_4$	14·45 g
$CaCl_2.2H_2O$	3·335 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	9·25 mg
$FeSO_4.7H_2O$	99 mg
stock salts solution	50 ml

The nitrilotriacetic acid is dissolved and neutralized with about 7·3 g KOH. After adding the remaining ingredients the pH is adjusted to 6·8. The stock salts solution contains per litre:

ethylenediamine tetra-acetic acid	2·5 g
$ZnSO_4.7H_2O$	10·95 g
$FeSO_4.7H_2O$	5 g
$MnSO_4.H_2O$	1.54 g
$CuSO_4.5H_2O$	0·392 g
$Co(NO_3)_2.6H_2O$	0·248 g
$Na_2B_4O_7.10H_2O$	0·177 g

A few drops of H_2SO_4 are added to reduce precipitation. The organisms are spot-inoculated onto surface-dried agar plates of yeast extract agar which is made by adding per litre: 5·0 g yeast extract and 20 g agar to the basal mineral medium. After good growth is obtained on the yeast extract agar, inocula from the colonies are transferred by a replica-plating procedure

onto plates of the test medium which contains per litre: the basal mineral medium constituents, 10 g Ionagar No. 2 (Oxoid) or some other purified agar, and 1 g of the carbon compound. A lower concentration of certain potentially toxic compounds is required. After incubation, the utilization shown by increased growth is compared with that on the control test medium lacking the carbon source.

This principle can be applied to tests of more fastidious organisms such as lactic acid bacteria if the yeast extract agar is replaced by a more complex medium (see Whittenbury, 1963) and if minimal requirements of growth factors and nitrogenous compounds are added to the test medium. If necessary, the test may be performed with anaerobic cultures.

The utilization of organic acids may be detected more readily by incorporating an appropriate pH indicator (e.g. phenol red, bromothymol blue) into a suitable medium. The breakdown of the acidic substrate brings about an easily detectable rise in the pH of the medium.

Liquid media may facilitate a more sensitive test, since agar, especially less highly purified brands, may provide alternative carbon sources that can promote the utilization of the organic acid. False positive results attributable to carry-over of traces of complex media in the inoculum are also less likely to arise in a liquid test medium than in an agar plate that allows localization of the spot-inoculum.

Tests that demonstrate the utilization of citrate and malonate are of use in the differentiation of certain members of the *Enterobacteriaceae*: In the test for citrate utilization the organisms are inoculated into Koser's liquid citrate medium (Koser, 1923) which contains Na citrate and supplies nitrogen in the inorganic form. Simmons (1926) added agar and an indicator to the medium and the composition, per litre, of a generally useful modification is:

sodium citrate	2 g
$MgSO_4.7H_2O$	0·2 g
NaCl	5 g
$NH_4H_2PO_4$	1 g
K_2HPO_4	1 g
1·5% alcoholic solution of bromothymol blue	10 ml
washed agar	20 g

The medium (pH 6·8) is made with distilled water and distributed as slopes after autoclaving. The cultures are lightly inoculated and should be incubated for 4–5 days at 37°C. A positive result is indicated by the development of turbidity in Koser's medium or by a blue colour on the medium of Simmons. A subculture from a positive test to a second test medium provides a check on false positives attributable to the carry-over of compounds in the initial inoculum.

An organism that produces negative results in Koser's or Simmons's media may or may not produce a positive result in a modified citrate medium of Christensen (1949) that affords a reliable test for citrate utilization in the presence of organic nitrogen. The medium contains per litre:

sodium citrate	3 g
glucose	0·2 g
yeast extract	0·5 g
cysteine monohydrochloride	0.1 g
KH_2PO_4	1 g
NaCl	5 g
phenol red	0·012 g
agar in distilled water	15 g

The pH is not adjusted. The autoclaved medium is dispensed in tubes as short thick slopes and is inoculated by a straight wire. The butt of the slope is stabbed and the wire is then drawn once up the slope. Tests are read after incubation for up to 7 days. A positive result is indicated by the development of a magenta colour; in negative tests, the medium remains yellow. An organism that produces a positive result in Koser's or Simmons's test media will also be positive in Christensen's test medium.

Malonate utilization is tested in a medium that contains per litre:

sodium malonate	3 g
NaCl	2 g
yeast extract	1 g
$(NH_4)_2SO_4$	2 g
K_2HPO_4	0·6 g
KH_2PO_4	0·4 g
bromothymol blue in distilled water	0·025 g

The pH is 7·4. The test organism is cultured in this fluid medium at 37°C for 2 days. If the acidic malonate is utilized, the indicator changes from green to blue, but it should be noted that in this test the sodium malonate is not the sole carbon source. The addition of 2 g DL-phenylalanine or 1 g L-phenylalanine to this malonate broth allows a combined test for malonate utilization and phenylalanine deaminase production (Shaw and Clarke, 1955). When the malonate test has been read, the medium is acidified with 0·1N NCl until it just turns yellow and about 2·2 ml of a 10% aqueous solution of $FeCl_3$ is then added. The mixture is shaken; the development of a dark green colour, which quickly fades, indicates that deamination has occurred.

Fermentation of tartrate, citrate and mucate are reactions of special use in classifying salmonellae. The tests require considerable attention to detail; they are described well by Edwards and Ewing (1962, p. 250) and Cruick-