METHODS IN MICROBIOLOGY

Volume 19
Current Methods for Classification and Identification of Microorganisms

Edited by

R. R. COLWELL

and

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PREFACE

The systematics of microorganisms has received a great deal of attention in recent years, particularly with the development of new methods for tracking the evolution of microbial species. The techniques for molecular genetic analysis have advanced substantially from the early work on DNA base composition to DNA/DNA hybridization and the more recent and exciting methodology of DNA and RNA sequencing. Coupled with these biochemical and molecular genetic advances are computer applications in systematics. Software for large scale data handling provides extraordinary opportunities for improved identification and classification of microorganisms.

This volume will provide an excellent means for initiating students into the excitement of microbial systematics, generated by the developments of the last few years. It is hoped that the volume will fill a need for a methodology text dealing with techniques for identification and classification of microorganisms The techniques covered range from those that are relatively simple, including classical and rapid identification methods to those used routinely in molecular biology as, for example, the most recent procedures of DNA and RNA sequencing.

Both the novice and the experienced research worker will be interested in some aspects of the material provided herein, which was intended to provide an "alpha and omega" coverage of the methodology for microbial systematics. Because one must "freeze" the knowledge available in order to review the state of the science at a given time, there is always the danger of the material rapidly becoming dated. In this case, the volume was planned to be both broad in scope and detailed in presentation, so that it will provide a valuable and useful reference, as well as a record of the field at the contemporary stage of development.

We would like to express our appreciation to Academic Press for the care with which they have prepared the material for publication and the high quality they have achieved.

April 1987

R. R. Colwell
R. Grigorova

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Classical and Rapid Identification Methods for Medically Important Bacteria

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METHODS IN MICROBIOLOGY
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I. Introduction

A. General considerations

In the early days of bacteriology, obtaining pure cultures of microorganisms from the mixtures in which they generally occur in Nature was the first important advance in distinguishing one kind of organism from the others. Using a solid medium and "streak plate" or "pour plate" methods, investigators were able to isolate and identify many kinds of bacteria. Early taxonomists considered mainly cell shape and staining, cell size, and the form of colonies, type of growth in broth, site of isolation, and pathogenicity. Subsequently, it has been recognized that cultural properties on simple media are too variable to be expressed as positive or negative characters and that habitat and pathogenicity are unsatisfactory features for a systematic classification. Even morphological and staining properties, which remain of great importance in taxonomy, are now regarded, in view of the availability of excellent selective and differential media, as not worthwhile to determine in routine identification of certain organisms, e.g. the Enterobacteriaceae. Early studies on nutritional, growth temperature and atmospheric requirements soon led to the discovery that Nature has produced a wide variety of physiological entities among the bacteria. Transformation of chemical compounds by bacterial enzymes is now recognized as an important tool in classification. Growth inhibitors are also widely used for both isolation and taxonomic characterization.

Thousands of identification methods have been described in the literature. The overwhelming majority represent modifications of tests discovered in the first half of this century, when the foundation of modern taxonomy was laid. Selection of the best of these methods or replacement of an old test with an improved one requires extensive comparative trials. In choosing methods, an enormous number of commercial reagents, dehydrated or complete media and multitest systems also have to be considered. In general, any method is satisfactory that gives accurate and reproducible results. Tests used in routine clinical and public health bacteriology should be suitable for a tolerably rapid and reliable identification.

In this chapter methods are described that may be considered as reference procedures (Kauffmann, 1954; Edwards and Ewing, 1972; Cowan, 1974; Holdeman *et al.*, 1977; Paik, 1980; Sonnenwirth, 1980; Vera and Power, 1980). In Hungary these methods have proved to give consistent results and have been prescribed as standard tests for the Hungarian Public Health Laboratory Service and for Hungarian hospital microbiology laboratories (Lányi, 1980).

Because of the limited space available in this chapter, identification

methods for mycobacteria have been omitted. These have been described in detail elsewhere (Vestal, 1975; Runyon *et al.*, 1980; Kubica and David, 1980) and have been used as reference methods all over the world. Determination of antigens and toxins also will not be described here, having been covered in earlier volumes of this series. Bacterial serology, although providing a practical and rapid aid for the identification of many bacteria, serves best for secondary subdivision of taxa.

B. Basic requirements of identification

To determine their characteristics, bacteria must be grown in pure culture, which is a group of organisms that have developed from a single cell or from a single clump of similar cells. In routine diagnostic work it is usually—and, for saving time, it has to be—sufficient to subculture a single colony grown on the primary plate. Taxonomic studies, in contrast, require several replatings from separate colonies, until an undoubtedly pure subculture is secured. Common sources of impure culture are colonies picked from the selective medium, which suppresses growth of most unwanted bacteria but does not kill them. On a selective plate seeded with specimens containing many kinds of microorganisms, inhibited but living cells are scattered throughout the surface of the medium and under the colonies of the non-inhibited organism. For subculturing, the culture that is sought must be picked up by touching only the point of an inoculating needle or loop on to the surface of one well-isolated and seemingly pure colony. It would be a serious mistake to "sweep" two or more colonies and/or to touch the seemingly "sterile" surface of the plate. Another source of impurity is the spreading growth of some bacteria, which can be avoided by using media inhibitory to swarming. A simultaneous subculturing of the colony on a differential plate, other than the primary one, saves time in repeating the purification process. For example, a suspected Salmonella colony from bismuth sulphite agar should be transferred, in addition to triple sugar iron agar and other tubed media, to brilliant green or MacConkey plates. Cultures that are difficult to identify are frequently mixed ones and should be examined for purity by streaking on several kinds of differential and selective media.

A correct incubation temperature is important for obtaining interpretable results. As a rule, tests should be done at the temperature optimal for growth, which may be estimated by observing growth of the culture on media seeded with the specimen. To permit growth of common pathogens and opportunistic pathogens, primary plates are usually incubated first at 35–37 °C overnight, followed by incubation for another day at 22–30 °C. Two temperature ranges are also sufficient for most identification tests: 35–37 °C for most

clinically important bacteria and 30 °C for glucose-non-fermenting, Gram-negative bacteria. The incubation temperature will be specified below only if different from that required for optimal growth.

The atmospheric requirement of isolates is evident from their growth on primary plates. Tests, unless otherwise stated, should be carried out under optimal conditions, i.e. in air (aerobes and facultative anaerobes), in air with 10% CO_2 (capnophilic bacteria), in nitrogen with 10% CO_2 and 5% O_2 (campylobacters and other microaerophilic organisms), or in nitrogen with 10% CO_2 and less than 1% O_2 (anaerobes). Tests for anaerobic bacteria include cultivation in anaerobic incubators and in ordinary anaerobe jars; the same media are suitable for pre-reduced preparation and handling (Holdeman *et al.*, 1977), a procedure that cannot be performed in most clinical laboratories because of lack of equipment more elaborate than anaerobe jars.

To obtain reproducible results, the incubation time is of great importance. With tests requiring culturing, a stage of sufficient multiplication, i.e. turbidity or precipitate in liquid media and good surface growth on solid media, should be obtained. To ensure adequate growth, the media should be supplemented with 5% serum and/or growth factors for fastidious bacteria and with 3–7% NaCl for halophilic vibrios. The standard incubation time for rapidly growing bacteria is specified for each test. For a tentative diagnosis in routine work, an incubation period shorter than that prescribed for a standard reading is usually sufficient, especially if the test is positive. For anaerobic bacteria the test should be read when good growth is evident or when no increase of growth occurs after an additional 8 hours of incubation (Holdeman *et al.*, 1977).

Growth is influenced by the size of the inoculum and the volume of the test medium. The inoculum, whenever different from the usual needlepoint amount of solid culture or 0.2–0.3 ml of anaerobic broth culture, is specified. Except when otherwise stated, the volume of the test medium is 2–3 ml for narrow tubes (diameter 10–12 mm) or 5–6 ml for ordinary test tubes (usual diameter 16 mm). For Petri dishes 90 mm in diameter, about 25 ml of medium should be used.

An essential point in identification is biological quality control. All batches of media and reagents must be tested with appropriate cultures before being put into routine use (see interpretation of individual tests). As reference ingredients of media, this chapter specifies commercial products that have been proved suitable from long personal experience. Other products, as far as they give interpretable and reproducible results, can be considered equally good.

II. Morphological characterization

A. Cell morphology

In practical identification of bacteria, cell morphology is determined either in unstained wet preparations examined by phase-contrast microscopy or in stained smears observed with ordinary optics. Wet mounts of young cultures reflect the natural morphology of the organisms, whereas in stained preparations, because of a shrinking of their protoplasm, bacteria appear shorter and thinner than their actual dimensions. Different procedures have been described by Norris and Swain (1971); this chapter presents tests used most frequently in routine identification.

1. Wet mounts

(i) *Procedure.* Mix a needlepoint amount of culture grown on solid medium in a small drop of water placed on an ordinary plain microscope slide and place a coverslip onto it. Cultures grown in liquid medium are suitable without dilution. The film of bacterial suspension should be thin; absorb the excess liquid with a piece of blotting paper so that the coverslip does not float over the slide. Examine immediately under a phase-contrast microscope with a high-power dry or an oil-immersion objective. The preparation, unless sealed with molten vaseline or with nail varnish along the edge of the coverslip, dries rapidly and becomes unsuitable for examination.

(ii) *Interpretation.* Record the shape and approximate size of cells. For motility examination see Section C.

2. Staining procedures

Stains described below are satisfactory for observation of the morphology and staining properties of most bacteria. The methylene blue stain serves for simple examination of cultures and is advantageous for presumptive recognition of bacteria in smears prepared from clinical specimens. The Gram method is the most important differential staining procedure. In young cultures of Gram-positive bacteria, crystal violet and iodine form a complex with cell constituents that is relatively insoluble in ethanol or acetone, whereas Gram-negative bacteria readily lose the violet stain and take the counterstain, which imparts another colour to the cells. Isolates of some taxonomic entities are Gram-variable, but the number of such species is not sufficient to affect the value of the Gram stain. Bacteria having a high concentration of lipids in their cells are difficult to stain by ordinary methods.

They stain readily, however, with hot fuchsin-containing phenol. When once stained, they retain the dye after treatment with acidified ethanol, i.e. "acid-fast bacteria". The Ziehl–Neelsen carbol fuchsin stain diluted 1:3–1:10 may be used for organisms that are not acid-fast but take up other dyes less readily (legionellae, spirochaetes).

To make a stained preparation, the bacterial suspension is smeared thinly on a slide. After allowing it to dry at room temperature for a few minutes, the smear is fixed by passing, specimen side up, through the flame of a Bunsen burner, three or four times in succession. Fixation solidifies the protoplasm of bacteria and causes them to adhere to the slide. The gentle heating of the slide is insufficient to kill some bacteria (spore-formers, mycobacteria), and the slides should be decontaminated after examination. After fixing, the smear is covered with the staining solution, then washed off with water. The smear is dried in air or by blotting it with absorbing paper and examined under oil immersion, with the oil placed directly on the stained smear.

(a) Methylene blue stain

(i) *Reagent.* Saturated alcoholic methylene blue solution (approx. 1.4 g dye in 100 ml 95% ethanol), 30 ml; potassium hydroxide, 1% aqueous solution, 1 ml; distilled water, 100 ml.

(ii) *Procedure.* Stain the fixed smear for 2 min, rinse with water and blot.

(iii) *Interpretation.* Cells stain blue.

(b) Gram stain (Hucker's modification)

(i) *Reagents. Crystal violet solution*: crystal violet, 2 g; ethanol (95%), 10 ml; distilled water, 90 ml. *Oxalate solution*: Na-, K- or NH_4-oxalate, 1 g; distilled water, 100 ml. *Lugol's solution* (for Hucker's stain): iodine, 1 g; potassium iodide, 2 g; distilled water, 300 ml; add a few ml of distilled water to the iodine and potassium iodide, mix, add small amounts of water gradually until the substances are dissolved, then bring up the volume to 300 ml; store in a glass stoppered bottle protected from light. *Decolourizer*: ethanol (95%). *Safranin solution*: safranin O, 0.25 g; ethanol (95%), 10 ml; distilled water, 90 ml.

(ii) *Procedure.* Suspend the cultures in small drops of water placed on a slide and prepare smears (not more than 5 on one slide 25 mm × 75 mm in size). Make the first smear round and the others oval and record their

position. Prepare next to them a control smear consisting of a mixture of known Gram-negative and Gram-positive bacteria. Fix and stain for 1 min with a mixture of 1 volume of crystal violet and 4 volumes of oxalate solution. Wash with water. Apply Lugol's solution for 1 min. Wash with water and blot dry. Decolourize by allowing ethanol to drip onto one end of the inclined slide so as to flow evenly over the smears until the solvent flows colourlessly from the slide. Wash with water, stain with safranin for 2–3 min, blot and examine.

(iii) *Interpretation.* Gram-positive: the bacteria are deep violet (*Staphylococcus aureus*). Gram-negative: the bacteria are pink to red (*Escherichia coli*). Decolourizing time is influenced by the water content of ethanol. Blotting of the slide before decolourization prevents an uncontrollable dilution of ethanol with water remaining on the slide, but prolongs the decolourizing time.

(c) Ziehl–Neelsen stain

(i) *Reagents. Carbol fuchsin solution*: basic fuchsin, 10 g; ethanol (95%), 100 ml; add 900 ml of 5% aqueous phenol solution, mix and filter through paper. *Decolourizer*: ethanol (95%), 97 ml; concentrated HCl, 3 ml. *Loeffler's methylene blue solution*: methylene blue, 1 g; ethanol (95%), 100 ml; potassium hydroxide, 0.01% aqueous solution, 100 ml; the stain improves with keeping.

(ii) *Procedure.* Flood the fixed smear with carbol fuchsin and heat with a flame periodically three times in 5 min until steam rises. Wash with running tap water and decolourize by flooding with several successive portions of acid–alcohol until it flows colourlessly from the slide. Wash, counterstain with methylene blue for about 30 s, wash again and air dry. To prevent contamination of acid-fast bacteria from one slide to another, do not immerse the slides in vessels containing the stain or decolourizing agent and do not blot the preparations.

(iii) *Interpretation.* Acid-fast bacteria are red (*Mycobacterium tuberculosis*), and other organisms and background material are blue.

B. Capsules

Some bacteria form a relatively large mass of slime layer around their cells. Owing to a low affinity for dyes, the capsule is usually not visible by ordinary

staining. By use of special staining, the capsules and the cells exhibit different colours (Norris and Swain, 1971; Cowan, 1974). For routine use, "negative staining" of live bacteria with India ink is most satisfactory.

(i) *Procedure.* Mix a needlepoint amount of bacteria grown on solid medium in a loopful of India ink placed on a slide. Prepare a thin film by pressing a coverslip on the drop and removing the excess liquid with a piece of blotting paper. Examine immediately under a phase-contrast microscope.

(ii) *Interpretation.* The capsule appears as a bright zone between the dark cells and the grey background (*Klebsiella aerogenes*).

C. Flagella

Flagella are the locomotor organelles of bacteria, consisting of whiplike appendages invisible under an optical microscope. The number, position and arrangement of flagella are fairly constant taxonomic features.

1. Phase-contrast microscopic examination of motility

The procedure described in Section II.A.1. is recommended for examining the motility of bacteria other than Enterobacteriaceae, which should be tested in semisolid agar (Section II.D.). Observation of swimming movement in a wet microscopic preparation is not sufficient to determine the arrangement of flagella, although polar flagellated bacteria show an apparently rapid darting motility, whereas peritrichous cells usually move at a lower speed. True motility should be distinguished from the oscillatory (Brownian) movement due to a continuous jostling of bacteria by molecules of the suspending fluid. Motility is best in young broth cultures incubated at 22–30 °C. Motility must be examined immediately after placing the culture on the slide. Light has an adverse effect, and the number of motile bacteria will decrease rapidly.

2. Leifson's flagella stain

Flagella can be seen under an electron microscope and by special staining in which an opaque substance is precipitated on their surfaces so as to increase their thickness above the resolution of optical microscopes. Staining of flagella requires a very careful and standardized technique, including extremely clean slides and as low amounts of background materials as possible, in order to prevent precipitation of the stain on the surface of the slide.

(i) *Reagents.* *Solution A*: basic fuchsin certified for flagella staining, 0.8 g;

ethanol (95%), 100 ml. *Solution B*: NaCl, 1.4 g; tannic acid, 3.0 g; distilled water, 200 ml. Mix the two solutions by thorough shaking. Allow to settle for 24 h, distribute in convenient amounts and store in the freezing compartment of a refrigerator. Just prior to use, warm the required amount of dye to room temperature and, if necessary, correct the pH by adding 0.1 volume phosphate buffer (0.5 M KH_2PO_4 and 0.5 M K_2HPO_4 mixed to provide a final pH of 6.0–6.2 to the dye solution). *Preparation of slides*: clean slides by boiling in 20% nitric acid for 5–10 min or by immersing them for 24 h in chromic–sulphuric acid (potassium dichromate saturated aqueous solution, 500 ml; concentrated sulphuric acid, 800 ml); wash by rinses of distilled water to remove all traces of acid, and allow to drain; store in a closed container.

(ii) *Procedure*. Use young cultures grown at 22–30 °C. Add 0.25 ml formalin (commercial preparation containing about 35% formaldehyde) to 4 ml broth culture, allow to stand for 15 min after gentle mixing, add about 4 ml distilled water, mix and centrifuge at 2000 rpm. Decant the supernatant, add about 8 ml distilled water, mix and centrifuge. Decant the supernatant and resuspend the bacteria in distilled water to a slight turbidity. Heat the slide in the blue portion of a Bunsen flame. Place a loopful of bacterial suspension at the end of the cooled slide tilted at about 80° and allow the liquid to flow lengthwise to the opposite end. Remove the excess with a blotting paper and dry at room temperature. Bacteria are fixed by the stain; do not heat the slide. Flood the slide with the stain. Staining lasts for 5–15 min, depending on the temperature and the age of the reagent: the higher the temperature of the laboratory, the more rapidly does ethanol evaporate from the stain and the dye precipitate, whereas an old solution stains less rapidly than a freshly prepared one. When staining is complete, a lighter red precipitate forms in the solution. Gently wash the stain off with water; do not pour the stain off before rinsing. Wipe the back of the slide and allow to dry in air.

(iii) *Interpretation*. Examine several areas of the slide and many flagellated bacteria for number and arrangement of flagella. Polar flagellated bacteria are regarded as monotrichous if the majority of cells have just one flagellum, and lophotrichous if the majority of cells have two or more flagella.

If the above method fails to give a sufficient number of flagellated bacteria, try to prepare a suspension as follows. Carefully pipette about 3 ml distilled water on nutrient agar slope cultures and allow them to stand until bacteria separate spontaneously from the agar and form a cloudy suspension; do not add formalin and do not centrifuge. Other flagella stains have been suggested by Cowan (1974).

D. Spores

The spore is a stage in the life cycle of certain bacteria, when the cell protoplasm becomes condensed into a small body surrounded by a relatively impervious wall. The spores may be spheroidal or ovoid in shape, and their position within the parent cell may be central, subterminal or terminal; they may or may not swell the organism. Of medical bacteria, *Bacillus* and *Clostridium* produce spores. Since there is a tendency for some species to lose the ability to produce spores, it is often necessary to cultivate suspected spore formers for up to 14 days on media enhancing sporulation (e.g. on garden-soil-infusion nutrient agar). Although ordinary stains do not penetrate the spore wall, the outlines of intracellular and free spores can be seen in stained films under conventional illumination. In wet preparations under a phase-contrast microscope (Section II.A.1.) spores appear as brilliant refractile objects. The use of spore stains (Norris and Swain, 1971; Cowan, 1974) is not necessary in identification.

E. Intracellular bodies

The presence of cytoplasmic inclusions of reserve material is an aid in the identification of certain kinds of bacteria. In medical bacteriology, the presence of metachromatic granules, also known as Babes–Ernst granules, is characteristic of most corynebacteria.

(i) *Reagents. Neisser's solution A*: methylene blue, 1 g; ethanol (95%), 20 ml; glacial acetic acid, 50 ml; distilled water, 1000 ml. *Neisser's solution B*: crystal violet, 1 g; ethanol (95%), 10 ml; distilled water, 300 ml. *Neisser's solution C*: chrysoidin, 2 g; hot distilled water, 300 ml; cool and filter.

(ii) *Procedure.* Stain the fixed smear for 5 min with a mixture of 2 volumes solution A and 1 volume solution B. Rinse with distilled water and counterstain with solution C for 10 s. Blot dry without washing. Prolonged counterstaining causes a fading of the metachromatic granules.

(iii) *Interpretation.* Metachromatic granules are dark violet-brown, cytoplasm is yellow (*Corynebacterium diphtheriae*).

III. Cultural characterization

A. Growth temperature and heat tolerance

As differentiating criteria, identification tables list, as a rule, growth at 4, 20, 30, 37, 41, 45 and/or 65°C. For testing, tubed media known to support the growth of the organism are inoculated lightly and incubated at different temperatures until good growth occurs in one or more of the tubes. For testing above 37°C, water baths should be used. In taxonomic studies the results should be confirmed by serially subculturing the organisms at the given temperatures several times.

Temperature tolerance means survival of the organism on heating. The test is used mainly for the differentiation of streptococci. One millilitre of Todd Hewitt or other suitable broth is inoculated with a drop of 24 h broth culture, placed in a water bath at 60 °C for 30 min, then incubated at 35 37 °C for 48 h. Growth indicates survival. *Streptococcus faecalis* is a useful reference culture for this test.

B. Oxygen and carbon dioxide requirement

In routine examination, the atmospheric requirement of an isolate can be estimated by considering the mode of incubation of the primary plates. For exact testing, inoculate a series of appropriate plated media and incubate the plates: (1) in an aerobic incubator; (2) in a closed incubator or jar with CO_2 content increased to 10%; (3) in a closed incubator or jar with CO_2 content increased to 10% and oxygen decreased to 5%; and (4) in a closed incubator or jar with anaerobic conditions. For description of anaerobic devices see Holdeman *et al.* (1977), Kaplan (1980) and Allen and Siders (1980). For a simple test of the ability of the culture to grow anaerobically, inoculate a tube of thioglycollate semisolid medium uniformly with a dilute saline suspension; facultative anaerobes grow throughout the medium, anaerobes below its surface and aerobes on its surface.

C. Nutritional requirements and growth factors

Bacteria display a remarkable diversity in the nutrients they can utilize. In systematic studies a wide variety of substances have been examined for utilization and growth-enhancing ability; however, few of these tests have been introduced into routine diagnostic medical bacteriology. Identification tables usually define growth on nutrient agar or in nutrient broth in the absence or in the presence of 5% serum or blood. Of growth factors, the