Nucleic Acid Techniques in Bacterial Systematics

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Series Preface

The science of microbiology owes its existence as well as its underlying principles to the talent and practical prowess of pioneers such as Leeuwenhoek, Pasteur, Koch and Beijerinck. Interest in microbiology has recently increased quite significantly given the exciting developments in genetics and molecular biology and the growth of microbial technology. There was a time when most microbiologists were acquainted with many of the techniques used in microbiology. It is, however, now becoming increasingly difficult for research workers to keep abreast of the bewildering range of techniques currently used in microbiological laboratories. This problem is compounded by the fact that scientists in any one field increasingly need to apply techniques developed in other scientific disciplines.

The series 'Modern Microbiological Methods' aims to identify specialist areas in microbiology and provide up-to-date methodological handbooks to aid microbiologists at the laboratory bench. The books will be directed primarily towards active research workers but will be structured so as to serve as an introduction to the methods within a speciality for graduate students and scientists entering microbiology from related disciplines. Protocols will not only be described but difficulties and limitations of techniques and questions of interpretation fully discussed.

In summary, this series of books is designed to help stimulate further developments in microbiology by promoting the use of new and updated methods. Both authors and the editor-in-chief will be grateful to hear from satisfied or dissatisfied users so that future books in the series can benefit from the informed comment of practitioners in the field.

MICHAEL GOODFELLOW

Preface

There has been a marked change in outlook in bacterial systematics over the past 20 years. The application of methods such as numerical and chemical taxonomy has greatly influenced our views on how bacteria should be classified and identified. It is, however, the development and application of nucleic acid based techniques that is revolutionizing current approaches to the classification, identification and characterization of bacteria. This book provides a comprehensive, up-to-date treatment of such methods and is written by specialists who have and continue to make significant contributions to this field of study.

The book deals with the applications of powerful techniques such as DNA and small RNA sequencing, gene amplification and DNA fingerprinting to bacterial systematics. The use of DNA and ribosomal RNA hybridization, DNA reassociation experiments and nucleic acid probes are fully explained, and basic methods for isolating and purifying nucleic acids are described in detail. We sincerely hope that the book will be of great value to microbiologists and microbial technologists interested in characterizing and identifying bacteria, and that it will encourage more young scientists to develop their talents in what is now a fascinating and rapidly developing area of biology.

The editors wish to express their gratitude first of all to the authors, who did a first class job and who submitted their manuscripts more or less on time. Thanks are also due to Patricia Sharp, Pru Theaker and Michael Dixon of John Wiley and Sons, for all of their help and encouragement during the preparation of this volume.

ERKO STACKEBRANDT
MICHAEL GOODFELLOW

Introduction

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A. BACKGROUND

Systematics, the scientific study of the diversity of organisms and their relationships, is a fundamental discipline that encompasses classification, nomenclature and identification. Classification or taxonomy is the ordering of organisms into groups (taxa); nomenclature is the assignment of the correct international scientific name to organisms; and identification is the placement of unknown strains into groups derived from classification. Sound classification is a prerequisite for stable nomenclature and accurate identification.

Most contemporary bacterial taxonomies are natural in the sense that they are based on overall resemblance. These taxonomies are sometimes called phenetic classifications since they are derived from similarities and differences in phenotypic features. In contrast to 'phylogenetic', the word 'phenetic' does not have any evolutionary implications, except within the context of showing the end product of evolution. Phylogenetic classifications are expressions of the evolutionary relationships between organisms. They reflect the degree of change in evolutionary lines.

There is no choice but to classify, identify, and name. At a purely practical level, microbiologists need to know what organisms they are working with before they can pass on information about them. An organism's name is the key to its literature, that is, an entry to what is known about it. For bacteria, this knowledge has traditionally been acquired using experimental and observational techniques since biochemical, chemical

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and physiological properties are usually required, in addition to morphological features, for the description of taxa. The practical value of taxonomies is shown by how they stand the test of time. Those found wanting will soon be ignored, modified or superseded by better classifications.

The basic unit in systematics is the species. A bacterial species can be considered as a group of strains defined more or less subjectively by criteria chosen by the taxonomist to show to best advantage and as far as possible put into practice an individual's concept of what a species is (Cowan, 1978). The number of species in a genus is influenced by the aims of the taxonomist and the criteria adopted to define species. There is no official or generally accepted definition of the term species in bacteriology. It can, however, be useful to distinguish a *taxospecies*, a group of strains of high mutual similarity, from a *genospecies*, a group of organisms capable of genetic exchange, and both of these from a *genomic species*, a group showing high DNA–DNA homology values. By assuming similarity between species, they may be arranged into genera, which may, in turn, be fused into higher taxa (e.g. families) until the whole range of variation is accounted for in the hierarchical system.

For practical reasons, classifications and nomenclature should remain stable. Changes can create confusion, particularly at genus and species level, and cause expensive modifications of identification procedures. Nevertheless, by their very nature, classifications tend to be transitory as they cannot always be modified to accommodate new information. Taxonomists would be failing in their duty if they were not to compile, for the benefit of the whole microbiological community, an information storage and retrieval system to reflect at any one time the current state of knowledge.

Historically, bacterial classifications were generated primarily for the purposes of identification. Organisms were assigned to groups based on morphology, staining properties, pigmentation, the presence or absence of spores, nutritional requirements, the capacity to produce acid from sugars, and the ability to grow in the presence of inhibitory compounds. In other words, they were derived very largely on behavioural properties of strains. This approach was practical since it was founded on characters that were easy to study and yielded information on what organisms did. Thus, early taxonomies were based on data relevant to the part played by bacteria in disease, food spoilage and soil fertility. However, the overreliance placed on small numbers of subjectively chosen properties, led to serious misclassification and dependence on inadequate characters for identification purposes (Goodfellow and Dickinson, 1985).

B. THE NEW SYSTEMATICS

Bacterial systematics has undergone revolutionary change in the past thirty years. A subject once widely perceived as boring and esoteric has developed into an exciting and rapidly developing scientific discipline that is changing our views on how bacteria should be classified, identified and characterized. The importance of the new bacterial systematics is recognized by microbial technologists searching for new products (Goodfellow and O'Donnell, 1989), clinical bacteriologists requiring improved identification and typing methods (Hawkey, 1989), and microbial ecologists monitoring the impact of the release of genetically engineered microorganisms on the indigenous microflora in natural habitats (Jain *et al.*, 1988). The beginning of this new era in bacterial systematics can be traced back to the introduction and application of new taxonomic concepts and techniques in the late 1950s and 1960s. Particularly important advances were derived from the use of chemical, numerical and molecular taxonomic procedures (Goodfellow and Minnikin, 1985; Goodfellow *et al.*, 1985).

Chemosystematics, or chemical taxonomy, is a rapidly expanding discipline in which information from chemical analyses of whole organisms or cell fractions is used for classification and identification, and for tracing evolutionary trends. Chemical features are increasingly being used to describe and separate bacteria, notably actinomycetes (Williams *et al.*, 1989). A wide array of chemical methods are now used to determine DNA base, lipid, wall amino acid and sugar, and whole-organism protein composition (Goodfellow and Minnikin, 1985; Gottschalk, 1985). In addition, rapid epidemiological typing of clinically significant bacterial pathogens can be achieved by the application of pyrolysis mass spectrometry (Freeman *et al.*, 1990).

Conventional numerical taxonomy has been the most effective method used to establish relationships below the genus level (Goodfellow and Dickinson, 1985; MacDonell and Colwell, 1985). In essence, numerical classification involves the generation of large data bases for many organisms, which are grouped into clusters (taxospecies) on the basis of shared similarities. Initially, all characters are given equal weight and, after reproducibility testing, used to generate probability matrices for the numerical identification of fresh isolates (Williams *et al.*, 1985). This approach is in sharp contrast to traditional practice in bacterial taxonomy, as taxa are defined and recognized using many equally weighted features, not on a small number of subjectively chosen behavioural, morphological and staining properties. Numerical classifications are based on phenetic data so that affinities between strains, and the hierarchies built upon them, are entirely phenetic.

It soon became apparent that reliable phylogenetic classifications of bacteria were not feasible using chemotaxonomic and numerical taxonomic methods. Reliance on chemical, morphological and physiological properties resulted in the assignment of bacteria to suprageneric groups, many of which were subsequently shown to be heterogeneous (Goodfellow, 1989). Indeed, it was only with the introduction of nucleic acid studies that suprageneric classification became a reality.

Johnson (1989) outlined several of the advantages to be gained by basing classifications on genomic relatedness:

(a) A more unifying concept of a bacterial species is possible.
(b) Classifications based on genomic relatedness tend to be stable, that is, they can accommodate new information.
(c) Reliable identification schemes can be prepared once organisms have been classified on the basis of genomic relatedness.
(d) Information can be obtained that is useful for understanding how various bacterial groups have evolved and how they can be classified according to their ancestral relationships.

The purpose of this book is to provide details of the experimental procedures that are currently being used and developed to derive information from nucleic acids for the classification, identification and typing of bacteria. All such investigations start with the isolation of deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA). Some of the methods used to overcome problems associated with the isolation of DNA are considered in Chapter 1. This contribution also includes protocols for the isolation of DNA and RNA.

C. CLASSIFICATION: HYBRIDIZATION AND SEQUENCING STUDIES

The measurement of the extent to which single-stranded DNA fragments from one bacterial strain reassociate or hybridize with single-stranded DNA from another strain has been used to determine nucleic acid sequence similarity or DNA homology. Experience over the last three decades has shown that DNA–DNA reassociation data are mainly relevant when investigating relationships within and between bacterial species. Generally, bacteria within the same genomic species have DNA homology values above 70% (Wayne *et al.*, 1987), although the exact level below which organisms are considered to belong to different species varies (Zakrzewska-Czerwinska *et al.*, 1988). DNA reassociation experiments

have helped to clarify relationships between many bacterial genera (Schleifer and Stackebrandt, 1983), but much remains to be done. Specific procedures recommended for the estimation of DNA homology values have been described (Johnson, 1985; Owen and Pitcher, 1985). The nature of reassociation and hybridization reactions and a comparison of the techniques used are given in Chapter 2.

Determination of relationships by measuring the extent of binding between ribosomal (r) RNA and rDNA cistrons is also an established method that has been used to unravel affinities between major prokaryotic groups. Comparisons can readily be made between nucleotide sequences of rRNA preparations from diverse taxa (De Smedt and De Ley, 1977; Schleifer and Kilpper-Bälz, 1987) as the base sequences of rRNA cistrons are more highly conserved than most of the genes forming the bacterial genome (Doi and Igarashi, 1965; Dubnau *et al.*, 1965; Moore and McCarthy, 1967). Ribosomal RNA–DNA pairing procedures have not been as extensively applied as DNA–DNA homology techniques but even so over 1000 representatives of 350 archaebacterial and eubacterial species have been examined in the past twenty years (Stackebrandt, 1988). The method has had an impact on the rearrangement of many genera, notably in the classification of Gram-negative bacteria (De Vos *et al.*, 1989). The principles underlying the most successfully used rRNA–DNA pairing techniques are considered in Chapter 3 together wtih some developments designed to facilitate isolation, labelling and hybridization procedures.

The major breakthrough in determining the evolution and phylogeny of prokaryotes came with the introduction of rRNA sequencing techniques. Initially, ribosomal cataloguing provided the most exacting way of detecting phylogenetic relationships amongst prokaryotes (Fox *et al.*, 1980; Fox and Stackebrandt, 1987). The period between the rise (Fox *et al.*, 1977) and decline (Lane *et al.*, 1985) of this technique saw revolutionary advances in bacterial phylogeny. Particular credit needs to be given to those who developed the general principles underlying the technique (Sanger *et al.*, 1965; Silberklang *et al.*, 1979), and to those who recognized its potential for elucidating phylogenetic relationships (Woese *et al.*, 1975; 1990a,b; Woese, 1987).

It has become evident that Gram-negative and Gram-positive bacteria are phylogenetically distinct, and that the latter form a phyletic line which divides into two branches that can be distinguished by DNA base composition (Stackebrandt and Woese, 1981). The actinomycete–coryneform bacterial line, which includes bacteria with a guanine (G) plus cytosine (C) content above about 55 mol%, can be separated from the low G + C content (below 50 mol%) *Bacillus–Clostridium–Streptococcus* branch. Several taxa previously associated with the actinomycetes clearly belonged to this second evolutionary branch. The genus *Eubacterium* is phylogenetically

related to *Clostridium, Kurthia* to lactic acid bacteria, and *Thermoactinomyces* to the Bacillaceae (Tanner *et al.*, 1981; Stackebrandt *et al.*, 1987). The cataloguing approach also led to the discovery of a third urkingdom (Woese and Fox, 1977), highlighted the existence of complex, structured ancient main lines of descent (Balch *et al.*, 1979; Woese *et al.*, 1985) and showed that phenotypic traits such as morphology were poor phylogenetic markers (Stackebrandt and Woese, 1981; Woese *et al.*, 1982; Stackebrandt *et al.*, 1988).

The need to extend these early studies to 23S rRNA and to obtain more information from 16S rRNA led to the development of reverse transcriptase sequencing of rRNA (Qu *et al.*, 1983; Lane *et al.*, 1985) and to the analysis of cloned rDNA Taq amplified by polymerase in the polymerase chain reaction (PCR, Saiki *et al.*, 1988). Protocols for RNA purification, reverse transcriptase sequencing, a compilation of eubacterial 16S and 23S rRNA sequence and amplified primers, and a number of previously unpublished 16S rRNA sequences are given in Chapter 6. Phylogenetic trees can also be generated from 5S rRNA sequence data (De Wachter *et al.*, 1985; Hori and Osawa, 1986; Dams *et al.*, 1986; Van Den Eynde *et al.*, 1989). Procedures for the rapid sequencing of 5S rRNA are described in Chapter 5.

The reverse transcriptase technique, a rapid and relatively easy way to determine rRNA sequences, will continue to be useful, as it samples populations of rRNAs actually transcribed and found in cellular ribosomes. It seems likely, however, that the PCR will replace standard methods for cloning and sequencing of DNA and RNA genes given its technical simplicity, speed and sensitivity to small amounts of DNA. The basis of the polymerase chain reaction mechanism, its application in microbial systematics and ecology, and comprehensive protocols for would-be practitioners are described in Chapter 7. Similarly, protocols for sequencing conserved DNA genes are given in Chapter 4.

The introduction and application of more powerful sequencing and related techniques of molecular biology will further facilitate the generation of molecular data for characterization and taxonomic purposes. The resultant information explosion will necessitate comparable advances in tree-building methods and in determining the accuracy and reliability of phylogenetic trees. Current discrepancies between topographies of phylogenetic trees derived from data acquired from one or two molecules need to be explained as do the strengths and weaknesses of using information from different nucleic acid species. Some of the present problems in deriving phylogenies can be attributed to unbalanced strain selection, but other factors include dependence on different data handling techniques and working hypotheses (Felsenstein, 1988; Olsen, 1988; Sneath, 1989). A brief description of the ways in which sequence data are handled is given in Chapter 10 together with a combination of some of the methods available

for tree construction, and a consideration of why it is difficult to reconstruct trees accurately.

D. IDENTIFICATION: PROBES AND TYPING

The advances in molecular biology and gene technology have been used to develop improved methods for rapid identification and typing of micro-organisms, notably bacterial pathogens. The new diagnostic methods are based on the molecular structure and specific characterization of nucleic acids, particularly of DNA.

It is now commonplace to detect specific fractions of DNA in bacteria by DNA hybridization. Labelled DNA probe is first denatured and then allowed to react with denatured unlabelled DNA from test bacteria. The unlabelled test DNA is usually obtained by lysing bacterial colonies on a filter. The labelled DNA probe binds to any complementary DNA sequence fixed on the filter. Bound DNA is detected from washed preparations by autoradiography or by some other reaction appropriate to the labelling used. The essential step is to prepare a DNA probe that will detect DNA sequences from the target organism but not from other bacteria. Similarly, rRNA probes can be designed to detect taxa at higher levels in the taxonomic hierarchy.

Nucleic acid probes have been prepared for bacterial identification (Cheverier *et al.*, 1989; Groves and Clark, 1987; Kuczak and Mordarski, 1989), for the detection and identification of bacteria in mixed microbial communities (Stahl *et al.*, 1988), and for the identification of single cells (De Long *et al.*, 1989). The theoretical and experimental considerations underlying the development and application of nucleic acid probes are considered in Chapter 8. The latter part of this contribution focuses on the use of rRNAs as targets for nucleic acid probes.

The detection of bacterial subspecies and biotypes is assuming greater importance not only for epidemiological and environmental studies but also for rapid and accurate characterization of patent and production strains. Cleavage of chromosomal DNA by a restriction enzyme and visualization of bands obtained after electrophoretic separation, restriction endonuclease analysis, have been used to good effect in epidemiological typing (Kuijper *et al.*, 1987; Renaud *et al.*, 1988), especially where conventional markers have been shown to be inadequate. Methods used to prepare genomic DNA for restriction analysis are given in Chapter 9, as are protocols for the electrophoresis of DNA restriction fragments. The value of DNA and RNA restriction patterns in molecular taxonomy and epidemiology are also covered.

E. RECONCILIATION OF APPROACHES TO BACTERIAL SYSTEMATICS

Bacterial systematics, which began as a largely intuitive subject, has become increasingly objective with the introduction and application of new taxonomic methods, notably the molecular methods considered in this book. These developments have led to the generation of improved taxonomies, the adoption of changes in nomenclature, and better identification procedures. It has become established that DNA–DNA hybridization is the gold standard for the designation of bacterial species, and a molecular definition of species has been recommended (Wayne *et al.*, 1987). It is also accepted that nucleic acid sequencing and hybridization studies currently provide the only sound basis for determining phylogenetic relationships among all bacteria.

The need to reconcile traditional and molecular approaches to bacterial systematics is of vital importance (Murray *et al.*, 1990; Wayne *et al.*, 1987). These workers consider that classification and nomenclature should agree with and reflect genomic relationships as far as possible and that all preconceived notions need to be re-examined within this context. They also recommend that distinct genomic species that cannot be separated from other genomic species on the basis of any known phenotypic property should not be named until some differential phenotypic property has been highlighted. Indeed, the integrated use of phylogenetic and phenotypic characters, that is, polyphasic taxonomy (Colwell, 1970), is considered necessary for the delineation of taxa at all levels from genus to kingdom. This means that descriptions of new genera should, wherever possible, include either sequencing or hybridization data. It is also desirable that all sequences from which phylogenetic and taxonomic conclusions have been derived should be published or accessible through data banks.

It can be expected that developments will continue to be made in the exciting field of molecular classification and identification. It is to be hoped that the strategies and working schemes included in this book will allow beginners to contribute to the new bacterial systematics.

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1

Isolation and Purification of Nucleic Acids

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A. INTRODUCTION

If there are any constants in the phylogenetic studies of nucleic acids, the isolation of the nucleic acids might be a candidate. All such investigations start with the isolation of deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA). Also, a lot more time is probably spent just isolating the nucleic acids than doing the interesting experiments. The isolation of nucleic acids can be considered as a scientific practice, but at times it seems to approach an art. As a result, there have been published many variations of a few basic methods. Some of the problems that have spurred the development of better isolation methods include the time involved, loss of DNA during the isolation process, contamination with polysaccharides and other cellular components, difficulties with cell disruption, and the degradation or partial degradation of the DNA or RNA.

The purpose of this chapter is to review some of the DNA isolation problems and the approaches that have been used by various investigators to solve them. Also some protocols for the isolation of DNA and RNA are included.

B. GROWTH AND CELL LYSIS

(i) Growth conditions and culture age

The usually rapid growth rates of bacteria, relative to other organisms, are of great advantage for the quick generation of cell material. However, the

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growth curves of bacteria vary greatly and must be considered in the DNA or RNA isolation strategy.

The medium of choice will be one that provides the best growth rate and extent of growth for a given group of organisms. Generally for chemoheterotrophs, a peptone–yeast extract base medium supplemented with an energy source, usually glucose, and a buffer system suffice. We have found that a 50 mM potassium phosphate buffer (pH 7.0) works well for many of the anaerobic bacteria and it is relatively inexpensive. Media for facultatively anaerobic and aerobic organisms may not require buffering if a minimal amount of acidic end products is produced.

The oxygen requirement is also an important consideration. Anaerobic bacteria usually grow well in freshly prepared medium, with a nitrogen-filled head space (Cummins and Johnson, 1971; Holdeman *et al.*, 1977). The CO_2 requirements of those that need it can readily be supplied by the addition of a sterile sodium bicarbonate solution at the time of inoculation (Holdeman *et al.*, 1977). Mixing of anaerobic cultures (by stirring bar or gentle shaking) may also be important, especially for non-motile organisms where the cells may tend to settle out. Facultative anaerobic organisms are usually grown under aerobic conditions (shaking) where less acidic fermentation by-products are produced. It is in fact very difficult to maintain aerobic conditions because removal of oxygen from the medium is more rapid than the absorption of oxygen from the atmosphere. This exchange is optimized by using small volumes of medium per flask (for example 250 ml per 2-liter flask), flasks with fluted walls, and fast shaking rates (for rotary shakers, 250–450 rpm). It is probably most difficult to supply the correct oxygenation levels to microaerophilic organisms. Here the medium is first equilibrated with a mixture of air and CO_2 and this mixture is supplied to the culture during growth (Smibert, 1981; Thompson *et al.*, 1988).

Early stationary stage is the preferred time for harvesting a culture, since at this point the extent of growth is near maximum and cell death is still at a minimum. The attention that one must give to the growth curve will depend on the organism. For many organisms, the cultures are rather stable in the stationary phase, such that an overnight or 24-hour culture will work fine. For other organisms, for example, some of the clostridia where they may switch into a sporulation mode, the DNA and RNA can go from being intact to essentially gone within a 1–3 hour period. Rapidly dividing Gram-positive cells tend to be more susceptible to lytic enzymes than stationary cells because the muramic acid content is lower in the growing zone of the cell. One also has to be careful about what appear to be slow-growing organisms. Some really do grow very slowly, for example, many mycobacteria. In other cases, apparent slow growth may really be just a low viable population density, where cells are continually

dying and there is a linear build up of non-nucleic acid-containing cell wall debris.

(ii) Cell disruption

The disruption of the bacterial cells can be a very frustrating technical problem, and at times it has perhaps dictated whether or not a group of organisms was investigated. Methods for disrupting cells include the addition of detergents, digestion with lytic enzymes, and physical disruption in some form. To make cells more susceptible to lytic enzymes, growth in the presence of antibiotics that inhibit cell wall synthesis and growth in the presence of cell wall component analogs, such as glycine or threonine, have been used.

1. Gram-negative bacteria

Although many Gram-negative bacteria will lyse in the presence of detergents only, others may lyse only partially or not at all. A preliminary incubation of the cells in the presence of lysozyme, which is an endoacetyl muramidase isolated from egg white, will provide much more uniform lysis. The enzyme is inhibited by high salt concentrations; therefore a suspending buffer consisting of 50 mM Tris-hydrochloride (pH 8.0), 1 mM sodium ethylenediaminetetraacetate (EDTA) and 0.25 M sucrose can be recommended. The sucrose stabilizes the resulting spheroplasts until the addition of the lysing detergent.

2. Gram-positive bacteria

There are several enzymes available for rendering Gram-positive bacteria susceptible to lysis with sodium dodecyl sulphate (SDS). In addition to lysozyme, which is the most commonly used, these are: *N*-acetylmuramidase, isolated from *Streptomyces globisporus*, which also cleaves the muramic acid backbone; lysostaphin, an endopeptidase isolated from *Staphylococcus* sp. K-6-WI, which is specific for the cross-linking peptides of other staphylococci; and achromopeptidase, a peptidase isolated from *Achromobacter lyticus*, which is also active on the cross-linking peptides.

(a) *Lysozyme.* We routinely use the same suspending buffer as for the Gram-negative organisms. The suspended cells can be concentrated 10–50-fold, depending on the amount of cell material. Add 1–3 mg/ml lysozyme and incubate at 37°C until the cells are susceptible to lysis by the detergent. Test for this by taking out small samples at various times and adding the lysing reagents in the proper ratios (see below for the various procedures).