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REVIEW

MICROBIAL CHEMOTAXONOMY

CHROMATOGRAPHY, ELECTROPHORESIS AND RELEVANT PROFILING TECHNIQUES

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1. INTRODUCTION

This review surveys current knowledge of microbial chemotaxonomy and analytical methods used in this field, most attention being paid to bacteria.

Workers in this field at first had a limited range of methods at their disposal, and some techniques came to be preferred to others. Thus, the fatty acid composition of bacteria has most often been studied by gas chromatography (GC) and the cellular protein pattern of bacterial cells by electrophoresis. However, a wide range of methods is now available for use in chemotaxonomy, varying from paper and thin-layer chromatography (TLC) to more recently developed methods such as high-performance liquid chromatography–mass spectrometry (HPLC—MS) and HPLC—fast atomic bombardment—MS. In addition to these instrumental methods, various non-instrumental or "classical" taxonomic methods are in current use, e.g., fermentation, serology and phage typing.

In this review interest is focused on modern instrumental analytical methods, non-instrumental methods being discussed briefly. A few methods of potential future value in chemotaxonomy are also considered.

1.1. Microbial taxonomy

Taxonomy includes the classification, identification and nomenclature of microorganisms. The purpose of classification is to bring existing organisms into a logical arrangement. Classification is directed against both previously classified and recently discovered organisms. Microorganisms are classified according to similarities and correlations are often calculated, followed by cluster analysis. Whereas morphology was a major characteristic at the outset of bacterial classification, biochemical, physiological and genetic features have

now assumed increasing importance. The traditional means of classifying bacteria was to characterize them according to morphological criteria, and then to arrange them according to intuitive judgement. Although intuition in classification has been abandoned, a great deal of subjectivity is still involved, e.g., in the description of a species, genus, family or order. It is our belief that chemotaxonomy, particularly by establishing specific chemical markers, may bring more objectivity into classification and identification. It is hoped that the discovery of specific markers may also make bacterial taxonomy more practical and reduce its academic character. Demonstration of specific markers may also lessen the effects of the variance that often appears between laboratories when performing physiological and biochemical tests for identification. The latter should always be carried out after classification. Fortunately, the importance of standardization has been recognized in bacterial nomenclature. The Approved List of Bacterial Names was started in 1980 [1].

Relatively few efforts have been made to classify microorganisms phylogenetically, in contrast to higher organisms, where classification is based largely on evolutionary evidence. In vertebrates or plants it has been possible to trace the evolution from one species to another by means of fossils. In microbiology, evolution is difficult to ascertain because representative fossil material is scarce. Nevertheless, ideas on bacterial evolution have been developed [2]. It is acknowledged that clones giving rise to unique and distinct groups of bacteria must have separated at various stages of procaryotic evolution [3]. In particular, DNA/RNA and DNA/rRNA hybridizations and rRNA oligonucleotide cataloguing and sequencing have increased our knowledge of bacterial evolution. 16S rRNA cataloguing has indicated that *Archaebacteria*, which comprise the methanogens, halobacteria, and thermoacidophiles, were among the earliest departures from the main stem of bacteria so far detected. Later, the eight or so major groups of photosynthetic or chemosynthetic bacteria designated *Eubacteria* arose. *Archaebacteria* and *Eubacteria* are now considered to be the two main kingdoms of procaryotes. However, it is unlikely that all possible procaryotic organisms have yet been observed and isolated for study, and even more primitive organisms than the *Archaebacteria* may be detected in the future.

1.2. Chemotaxonomy

Chemotaxonomy was recently defined as the study of chemical variation in living organisms and the use of chemical characters in classification and identification [4]. Chemotaxonomy is not a new subject, as most bacterial taxonomies, on closer inspection, are based on chemical properties. In a more restricted sense of the word, chemotaxonomy deals with the distribution of amino acids, sugars, lipids, proteins and other substances in whole bacterial cells, parts of cells or fermentation products, and with enzymes. Chemotaxonomy made a large step forward with the introduction of GC and starch gel and polyacrylamide gel electrophoresis. A wide range of techniques, some of them complex and advanced, are available for studies on the chemistry of microbial cells. Integrated chemotaxonomic procedures establishing profiles of lipids, wall amino acids and sugars from the same biomass have been developed,

and increasing emphasis has been placed on automated systems and data analysis in routine work. Efforts have been made to achieve rapid classification and identification of bacteria from minute amounts of biomass, such as a single bacterial colony [5]. Obviously, each method has its strengths and weaknesses. A combination of techniques will often be of greater value than any single technique. Chemotaxonomy provides a better classification of bacteria, establishes degrees of correlation between them, traces aspects of their evolution and identifies clinically important organisms. Recent reviews dealing with these aspects include refs. 6—13.

2. BACTERIAL PRODUCTS AND PREPARATIONS FOR CHEMOTAXONOMY

2.1. Volatile and metabolic products

Whereas fermentation end products of aerobic eubacteria are usually inadequate for taxonomic purposes [4], metabolic end products of anaerobic bacteria (acids, alcohols and ketones) are very valuable. Acidic end product analysis is regarded as one of the essential biochemical tests for the characterization of anaerobes [14—16], and detailed patterns of acidic end products from anaerobic bacteria have been presented [14]. Various fermentation groups have proved to be of particular taxonomic value, e.g., lactic, ethanolic, mixed acid, butanediol, butyric, butylic, acetone and propionic fermentations [11]. Fermentation products such as acetic acid, isobutyric acid and isovaleric acid were demonstrated by means of headspace gas chromatography (HSGC) in *Bacillus cereus* and other *Bacillus* species [17]. The presence of D(−)-lactic acid and the ratio of L(+)- to D(−)-lactic acid may be useful taxonomic criteria for the study of Gram-negative anaerobic bacteria [18]. Even neutral fermentation products may assist in the characterization of aerobic and anaerobic bacteria and fungi. This was demonstrated by HSGC for a wide range of bacteria and fungi that produced neutral products during fermentation [19]: *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus faecalis, Streptococcus milleri, Streptococcus mitior, Streptococcus mitis, Streptococcus salivarius, Streptococcus sanguis, Escherichia coli, Klebsiella pneumonia, Klebsiella oxytoca, Proteus mirabilis, Morganella morganii, Serratia* spp., *Enterobacter cloacae, Bacteroides fragilis, Clostridium perfringens, Candida albicans, Candida krusei* and *Torulopsis glabrata.* In this experiment there were strains of *S. epidermidis* that produced or did not produce ethanol, which could be used as a chemical marker.

Amines, often regarded as basic end products, occur as metabolites from anaerobic and facultative bacteria, and the importance of amines in chemotaxonomy has been demonstrated for a number of organisms, e.g., *Proteus, Clostridium* [20—25] and others [26, 27]. HSGC of cultures from all *Proteus* species examined demonstrated the production of trimethylamine from acetylcholine [27]. The latter can be used as a marker for the detection of *Proteus* species. The biotransformation of exogenous substances is discussed more extensively in Section 6.1. Enterobacteria such as *Escherichia, Klebsiella, Citrobacter* and *Proteus* species from urine specimens were analysed by HSGC [28], the production of ethanol, methanethiol, dimethyl disulphide and tri-

methylamine from exogenous products being used as criteria. GC—MS analyses of volatile amines produced by *Clostridium* spp., *C. ghoni*, *C. bifermentans*, *C. sordellii*, *C. lituseburense*, *C. mangenotii*, *C. histolyticum*, *C. perfringens*, *C. difficile* and *C. cadaveris* demonstrated the formation of trimethylamine, isobutylamine and 3-methylbutylamine. In addition, methylamine was demonstrated in *C. sordellii*, *C. lituseburense*, *C. mangenotii*, *C. histolyticum* and *C. perfringens*. Dimethylamine was not detected in *C. cadaveris*. Ethylamine was absent from *C. sordellii* and *C. mangenotii* [20].

Polyamines have also been separated by capillary GC [29]. Polyamines are not as volatile as common amines. Improved separation systems for the determination of polyamines and related compounds by reversed-phase HPLC have been described [30]. With these systems polyamines, monoacetylspermidines, monoacetylputrescine, putreanine and isoputreanine were separated. The analysis of polyamines in methanogenic bacteria is a suitable chemotaxonomic method that corresponds well with the results obtained by 16S rRNA cataloguing [31].

Several related techniques exist for analysis of volatile metabolic products, e.g., direct column injection of the culture medium, extraction of samples with organic solvents, HSGC and on-column injection. In direct column injection of the culture medium the bacterial cells are usually removed beforehand. For the analysis of free fatty acids, several coating materials have been used, e.g., free fatty acid phase (FFAP) [32,33] and non-polar materials [34,35], which are sufficiently stable and give satisfactory separations. Direct injection of aqueous samples has been described [36]. A technique allowing the direct analysis of up to thirteen amines in aqueous solution has been developed by Tavakkol and Drucker [22]. Direct injection techniques do not allow samples to be concentrated and minor products may therefore remain undetected.

Extraction with organic solvents is used to exclude water from the sample and to concentrate the substances to be analysed. This method permits the selective extraction of acids and bases. Neutral components can be found as contaminants in both fractions. HSGC is an attractive method, able to analyse volatile substances of bacterial origin, e.g., acids, alcohols and amines. It is simple, rapid and suitable for large analytical series, but limited to typical volatile substances. Capillary columns are difficult to use owing to the low gas flow-rate. The problems of broad peaks and unsatisfactory separations due to the large volumes injected (1—3 ml) may be reduced by using split injection. The alternative to HSGC is the on-column technique, which has many of the advantages which the splitless headspace technique does not have. The sensitivity and separations may be improved if cold trapping is used [37]. The on-column technique has the drawback that all substances injected enter the column, so non-volatile substances may sediment in the injection part of the column, particularly when a mixture of volatile and non-volatile substances is being examined. The on-column technique is particularly suitable for the examination of thermolabile and chemically unstable substances. Evaporation takes place at a low temperature, and there is no contact between the substances and the metal surface. Splitless and on-column headspace techniques are more sensitive than the usual split-stream technique, but require more training and accuracy of the operator. The GC—MS of volatile substances has

advantages over the usual headspace technique [20,38], but requires expensive equipment and a qualified operator.

Analyses of metabolic end products by means of HPLC and isotachophoresis may be highly rewarding. With isotachophoresis, nanomole levels of acidic end products can be detected [18]. Acid metabolites from microorganisms have been analysed directly by HPLC without derivatization [39], and semiquantitative profiles of microbial metabolites including aliphatic, dicarboxylic and phenolic acids were obtained. Problems related to use of HPLC for the analysis of microbial organic acids are discussed in more detail in Section 3.3.4. Proton "spin-echo" NMR spectrometry is one of the latest developments in the analysis of metabolic end products [18]. Fermentation assays require careful standardization of the growth conditions, media and analyses.

2.2. Extracts

Extracts were probably the first preparations to be studied in analysis on bacterial chemistry. They are complex mixtures containing impurities from which the substances of interest will have to be isolated and often concentrated. Three different solvents provide almost similar extraction products: trichloroacetic acid, perchloric acid and ethanol. Ethanol is preferable when acid-labile substances are to be extracted. Extracts may contain capsular polysaccharides, polyphosphates, pigments, nucleotides, nucleosides, nucleotide sugars, flavins and other isoenzymes, amino acids or tricarboxylic acid cycle intermediates.

Aqueous extracts from bacterial cells are used to isolate amino sugars. Capsular polysaccharides and water-soluble oligosaccharides can be recovered from the aqueous solution. During extraction with water, cellular enzymes are deactivated if the aqueous suspension is heated to 100°C. Aqueous extracts are not contaminated with solvents but contain inorganic salts from the bacterial cells. Hot water is preferable to cold water when capsular polysaccharides are to be extracted. Hot water extraction yields a material highly contaminated with water-soluble oligosaccharides and other soluble polysaccharides and, to some extent, nucleic acids. Contamination with nucleic acids is due to autolysis of bacterial cells during growth and preparation. Such contaminants can be found in both hot and cold water extracts. Aqueous 1% (w/v) sodium hydroxide solution is also used for the extraction of capsular polysaccharides. This method may cause degradation of polysaccharides, particularly those with a large content of acetyl and pyruvyl groups, and tends to release intracellular contaminants, one of which is glycogen. Body fluids can be regarded as aqueous extraction fluids. When these fluids become infected and microorganisms multiply within them, bacterial metabolites, in addition to auto- and immunolysates, will be present. The same situation occurs in aqueous bacterial media. Analyses of infected media and body fluids have been reviewed [12].

Carotenoids may be extracted in the free form or as carotenoid glycosides. Carotenoids are very labile substances, which should be protected from light and oxygen to prevent isomerization and oxidation. They can be extracted

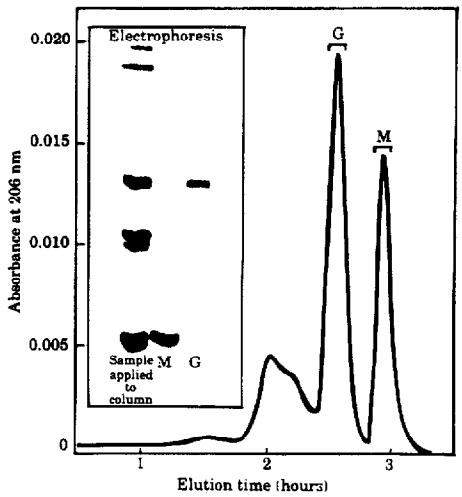

Fig. 1. Purification of viral glycoproteins by means of high-performance gel permeation chromatography. Sample: vesicular stomatitis virus (VSV) envelope proteins, labelled with [^{35}S]Met (31 μg in 20 μl), incubated with 80 μl of eluent containing 0.5% SDS for 5 min at 96°C. Eluent: 0.1 *M* Na_2HPO_4—NaH_2PO_4, 0.1 *M* NaCl, pH 6.8. Flow-rate: 90 μl/min. Detection: 206 nm, 0.05 a.u.f.s. Injection: 100 μl. Temperature: 30°C. (Courtesy LKB Produkter AB, Bromma, Sweden.)

with acetone. Some extracts may contain quinones and ferrodoxins, which also can be extracted directly with acetone. Carotenoid glycosides, which are extracted with methanol, must be saponified for isolation of carotenoids. Isooctane extraction, as performed for quinones, is an extraction method similar to that with acetone. The water content in bacterial cells is very critical when isooctane is used and lyophilized bacterial cells are therefore required. Proteins can be extracted in aqueous buffers such as acetate (pH 5) or citrate (pH 6). Extraction with urea in water has also been used. Acidic extracts usually contain haemoproteins, other proteins and nucleic acids. Before the proteins can be extracted from whole cells, the cell wall has to be destroyed. Cell destruction is unnecessary when surface proteins are to be extracted. Protein extracts can be purified by electrophoresis or chromatography (Fig. 1). Chromatofocusing has often been used in analyses of proteins and enzymes. For example, the separation of β-lactamase from *Enterobacter cloacae* [40] and *Escherichia coli* [41] using this technique has been described. The separation of peptides on a polystyrene resin column was described by Sasagawa et al. [42].

2.3. *Whole defatted cells*

These preparations, obtained by removal of free and bound cellular fatty acids [43], are intermediates between whole cells and isolated cell membranes. Whole defatted cells provide simpler gas chromatograms than do whole cells [44,45] and are suitable for sugar analyses. Lipid-free biomass is used as a starting material during the analysis of wall amino acids [46]. Methanolysed and trifluoroacetic anhydride derivatized whole defatted cells provided sugar patterns in the closely related *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*, which permitted precise GC differentiation [43].

When whole cells are used in chemotaxonomy, defatting can be recommended as a modification of cells that may facilitate the classification and identification of relatively unknown bacteria.

2.4. Whole cells

Analysis of whole cells has been used extensively for the classification of bacteria (e.g. ref. 47). The GC of methanolysed and trifluoroacetic anhydride derivatized whole cells provides a multitude of peaks [44,45] (Fig. 2), the identification of which may be difficult, especially when the constituents of the bacterial cells are unknown. However, for differentiation between closely related bacteria, e.g., members of the *Actinobacillus—Haemophilus—Pasteurella* group, whole-cell methanolysates are suitable in the routine clinical laboratory [44,45]. Whereas the sugar patterns of whole cells from these bacteria provided clear separations, their cellular fatty acids were of limited taxonomic value [45].

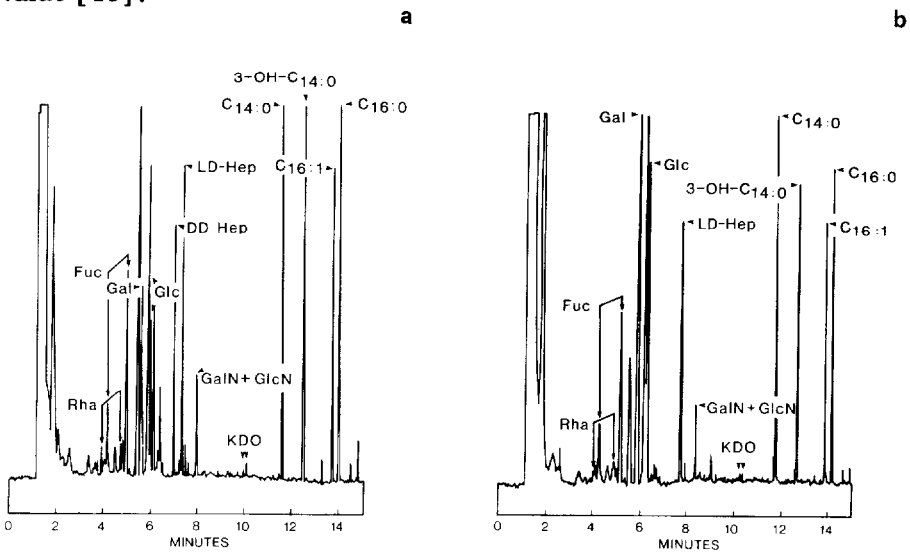

Fig. 2. Gas chromatograms of methanolysed and trifluoroacetic anhydride derivatized whole cells from (a) *Actinobacillus actinomycetemcomitans*, as represented by strain ATCC 33384, and (b) *Haemophilus aphrophilus*, as represented by strain ATCC 33389. Abbreviations: Rha = rhamnose; Fuc = fucose; Gal = galactose; Glc = glucose; DD-Hep = D-glycero-D-mannoheptose; LD-Hep = L-glycero-D-mannoheptose; GalN = galactosamine; GlcN = glucosamine. (Courtesy J. Chromatogr.)

2.5. Single colony analyses

Single bacterial colonies can now be analysed directly for their chemical components by GC. Brondz [13] recently developed a method by which a single colony from *Haemophilus aphrophilus* could be analysed for its sugar and fatty acid content by capillary GC (Fig. 3). With this technique the same sugars and fatty acids were detected as had previously been found with liquid-grown cultures [44]. There are several advantages of applying single

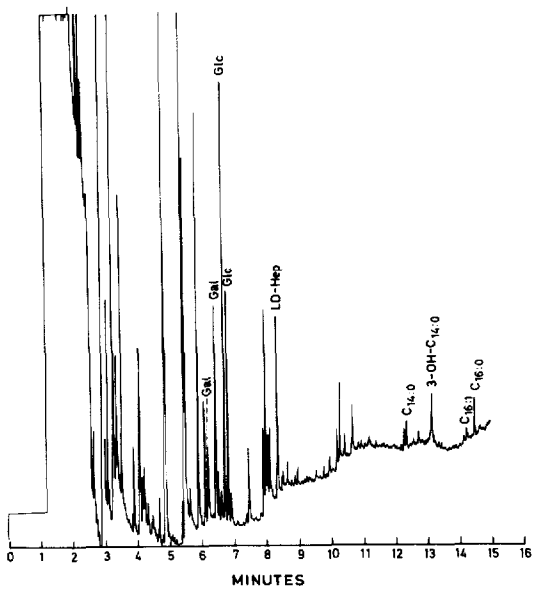

Fig. 3. Gas chromatogram of a single methanolysed and trifluoroacetic anhydride derivatized colony from *Haemophilus aphrophilus*, strain ATCC 33389. Abbreviations: Gal = galactose; Glc = glucose; LD-Hep = L-glycero-D-mannoheptose; $C_{14:0}$ = myristic acid; 3-OH-$C_{14:0}$ = β-hydroxymyristic acid; $C_{16:1}$ = palmitoleic acid; $C_{16:0}$ = palmitic acid. (Courtesy J. Chromatogr.)

colony techniques to liquid-grown cultures in chemotaxonomy [5]. Thus, mixed cultures can be analysed by means of single colonies. Solid culture analyses are also attractive owing to the speed by which they can be performed and the small biomass required. A fingerprint library has been developed by Hewlett-Packard, using a loopful of colonies, for the analysis of fatty acids from approximately 6000 different bacterial strains (Hewlett-Packard 5898A Microbial Identification System).

2.5.1. Pyrolysis—gas chromatography and —mass spectrometry

Bacterial colonies may also be analysed directly by pyrolysis—MS, but this depends on the organism [48]. Pyrolysis involves the breakdown of bacteria in an inert atmosphere using heat to produce a series of volatile, low-molecular-weight substances [48]. In pyrolysis—MS these fragments are detected and quantitated. Pyrolysis—MS, which is at an early stage of development, has been used for the characterization of mycobacteria [49,50] and yeast species [51, 52]. Shute et al. [48] used pyrolysis—MS to discriminate between four closely related strains of *Bacillus*. Basically, pyrolysis—MS involves the examination of the total cellular composition of microorganisms and as little material as a single bacterial colony can be analysed.

The development of pyrolysis—GC, particularly with exact temperature control by means of pyroprobes [53], has made it possible to obtain reproducible pyrograms [54]. Simultaneously, data analysis of pyrograms has been performed [55]. Pyrolysis—GC can be used, e.g., to recognize fungi. Thus, the combination of pyrolysis—GC and SIMCA pattern recognition gives a good classification [56]. Pyrolysis—GC has also been used to classify fruit bodies of

Ectocorrhizal suillus species [57] and to determine the fatty acid content of bacteria under classification [58]. Progress in this field has been reviewed [59].

3. CELL ENVELOPE COMPONENTS FOR CHEMOTAXONOMY

A great variety of substances in the bacterial cell envelope may be used for chemotaxonomic purposes [4]. In the plasma membrane such components include isoprenoid quinones, lipid-soluble pigments, lipoteichoic acids and analogues, polar lipids (fatty acids), isoprenoid ethers and other long-chain components. Bacterial cell walls contain possible chemotaxonomic markers such as proteins, murein and analogues, polysaccharides and teichoic acids and analogues. Further, the outer membrane of Gram-negative bacteria contains lipopolysaccharides and polar lipids, and the outer membrane of Gram-positive bacteria may contain bound lipids (mycolic acids) and free lipids (glycolipids, sulphoglycolipids and waxes).

3.1. Sugars

Bacterial sugars should be used more extensively in chemotaxonomy than has been done previously. Lipopolysaccharide (LPS) and capsular polysaccharides are the preparations most often examined. Sugar constituents of LPS of considerable taxonomic importance include neutral sugars, branched sugars, O-methyl sugars, amino sugars and acidic sugars [60]. Brondz and Olsen [44, 45,61] were able to use D-glycero-D-mannoheptose as a taxonomic marker in LPS and whole cells from *Actinobacillus actinomycetemcomitans* in its differentiation from *Haemophilus aphrophilus*. Aldoheptoses are almost unique for bacteria. The sugar patterns of whole cells from *Acinetobacter*, *Moraxella* and *Neisseria* were examined by Jantzen et al. [62], who found L-glycero-D-manno-heptose only in the genus *Neisseria* and "*Moraxella*" *urethralis*. Neuraminic acid distinguished strains of *N. meningitidis* groups B and C from other meningococcal serogroups. *Acinetobacter* contained several types of capsular polysaccharides. Capsular polysaccharides have also been well characterized in *Enterobacteriaceae* and *Neisseria*.

Aerobic actinomycetes have four different whole-cell sugar patterns [63]. Pattern A organisms contain arabinose and galactose, whereas xylose and madurose are absent. Pattern B organisms have madurose, but not arabinose, galactose or xylose. Pattern C organisms possess none of these characteristics, and those of pattern D contain arabinose and xylose, but lack galactose and madurose. Pattern A is characteristic of the mycolic acid-containing taxa *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus*, in addition to *Micropolyspora* and *Saccharopolyspora* [64]. *Actinomadura* and *Dermatophilus* exhibit sugar pattern B, *Nocardiopsis*, *Streptomyces* and *Thermoactinomyces* either lack all of the diagnostic sugars (pattern C) or have no characteristic sugars. *Micromonospora* is a good example of an actinomycete taxon of sugar pattern D. Sugar patterns of the fermentative actinomycetes are less specific and more variable than their oxidative counterparts. Facultatively anaerobic actinomycetes such as *Actinomyces* and *Rothia* have no detectable amounts of arabinose, xylose or madurose.

Corynebacterium matruchotii is the only filamentous, fermentative species with arabinose in whole-cell or cell wall hydrolysates. Other sugars such as 6-deoxytalose, fucose, galactose, glucose or rhamnose can also assist in the separation of these genera, and occasionally their species [64]. Characteristic sugar patterns have also been found in *Actinomyces bovis*, *A. israelii*, *A. naeslundii*, *A. odontolyticus*, *"A. suis"/A. denticolens* and *A. pyogenes*.

The presence of sugars and diaminopimelic acid permits unknown bacterial isolates to be classified according to cell wall chemotype [65] without the need to obtain purified cell wall samples.

Sugar analysis may be carried out after polar lipid and fatty acid extraction through acid methanolysis [44]. In *Actinomyces* methanolysis releases bound lipids such as mycolic acids and hydroxy acids as methyl esters and sugars as O-methyl glycosides [46]. Sugars are often analysed as alditol acetates or as alditol nitriles [11]. Partially methylated alditol acetates are used for structural analyses of polysaccharides. Bacic et al. [66] described the use of a vitreous-silica capillary column for the analysis of partially methylated alditol acetate. In recent years trifluoroacetic acid anhydride derivatives have found increasing application (e.g., refs. 46, 47 and 61). The trifluoroacetic anhydride procedure was recently used to analyse a single bacterial colony [5]. Methylation analyses of complex carbohydrates have been reviewed by Lindberg and Lønngren [67]. Structural analyses of sugars should be effected by MS [68]. The MS of hexosamines has been described in detail by Kochetkov et al. [69]. Brondz [13] described the MS of O-methyl trifluoroacetic anhydride derivatives of D- and L-glycero-D-mannoheptose. Carbohydrates were earlier considered as stable substances that provide little or no degradation products in the depolymerization of complex poly- or oligosaccharides. Recently, complex carbohydrates have been shown to disintegrate markedly during depolymerization [70]. Other techniques such as NMR spectrometry also have great potential in the elucidation of complex carbohydrate structures [71]. Structural analyses of bacterial sugars require a large input of resources and time and are therefore not applicable in the routine clinical laboratory, and composition analyses of complex carbohydrates are therefore most frequently used.

3.2. Teichoic/teichuronic acid

Teichoic acids are found instead of, or in addition to, polysaccharides in the cell walls of many Gram-positive bacteria. Two groups of teichoic acids have been detected: cell wall teichoic acids, which are covalently linked to murein through muramic acid, and lipoteichoic acids, covalently linked to lipids and associated with the plasma membrane [4]. As wall teichoic acids differ more in structure than do lipoteichoic acids, the former are most valuable as chemical markers. Teichoic acids are water-soluble polymers containing the polyols glycerol, mannitol or ribitol linked through phosphodiester bridges and substituted by amino sugars, sugars or D-alanine. Teichoic acids are immunologically active, and teichoic acids of different structure and serology have been used in the classification of staphylococci and lactobacilli. The occurrence of teichuronic acid, which generally is limited in bacteria, may assist in the identification of *Micrococcus luteus*, *Bacillus subtilis*, *B. licheniformis*,

B. cereus [72] and staphylococci [73]. Teichoic acid analysis has been described by Drucker [11].

3.3. Lipids and their constituents

Lipid classes in the cell envelope of bacteria may serve important chemotaxonomic purposes. Whereas the cell wall lipids of Gram-positive bacteria are concentrated in the plasma membrane, Gram-negative bacteria have lipoproteins, lipopolysaccharides (LPS) and polar and non-polar lipids located in the plasma and outer membrane. Certain Gram-positive bacteria, particularly mycobacteria, have a well defined outer membrane consisting of bound and free lipids. The lipids of eubacteria can be divided into a number of classes: polar lipids, neutral lipids, cyclic neutral lipids and fatty acids [74]. Some of these lipids have a clear chemotaxonomic potential, permitting the separation of specific bacterial groups, genera or species. In the biomembranes of eubacteria, phospholipids, which consist of the diacylglycerol phosphate type of lipid, are the major lipids. Phospholipids are the most common polar lipids in bacteria. Phyletic differences exist in the distribution patterns of phospholipids between specific bacterial groups. Archaebacteria differ from eubacteria by lacking acylglycerol-ester lipids. These lipids are also absent from the thermophilic anaerobe *Thermodesulfobacterium commune* [75], which is not an archaebacterium. It is also possible to distinguish between archaebacteria, i.e., halophiles, methanogenes and thermophiles, by the structure of the core diether and tetraether lipids [36]. Bacterial groups based on these polar lipid patterns correspond to those established by rRNA—DNA homology studies [4]. In archaebacteria, glycolipids and phospholipids are the polar lipids. These have sugar and phospho derivatives, as in most bacteria, except that the isoprenoid-chains are ether-linked to glycerol. The presence of specific ether lipids in *T. commune* suggests that this organism developed simultaneously with the archaebacteria. The absence of isoprenoid side-chains and the presence of alkyl hydrocarbons that are structurally common side-chains in eubacterial lipids differentiate *T. commune* from archaebacteria and eubacteria [74].

Nitrogen-containing lipids in bacteria include phosphatidylethanolamine, phosphatidylcholine and/or phosphatidylserine types [76]. Phosphatidylinositol mannosides of actinomycetes and related bacteria have great diagnostic value [77]. Similar lipids are the O-aminoacylphosphatidyl glycerols frequently seen in Gram-positive bacteria [76], N-acylphosphatidylserine of *Rhodopseudomonas* [78], the ornithine-containing lipids and the sphingolipids [79]. Ornithine-containing lipids have been isolated from a number of bacteria [74]. The presence of such lipids in *Bordetella* species and their biological significance have been described [80,81]. Sphingolipids are characteristic of members of *Bacteroides*. Sulpholipids are common in procaryotes. Thus, sulphonoquinovosyldiacyl glycerides have been found in photosynthetic bacteria, blue-green bacteria and eukaryotic photosynthetic bacteria, and in *Bacillus acidocaldarius* [82—84]. In *Capnocytophaga*, N-acylated and non-N-acylated 2-amino-3-hydroxy-15-methylhexadecane-1-sulphonic acids are major cell components that differentiate these organisms from all other bacteria [85].

The glycolipids are widely distributed in eubacteria and archaebacteria, but not to the same extent as the phospholipids [74]. Nevertheless, glycolipids are promising taxonomic markers. The structures of some glycolipids are species specific [82,86—89]. A new glycolipid has been found in *Bacillus acidocaldarius* [82], which contains a pentacyclic triterpenoid. The isolation, separation, identification and structure analysis of polar lipids have been reviewed [90].

Neutral lipids of bacteria tend to vary more and to be more species specific than polar lipids. Non-isoprenoid, acyclic hydrocarbons of *Micrococcaceae* differentiate these organisms from almost all bacteria, in addition to differentiating genera within *Micrococcaceae* [91]. Tetrads of methyl-branched acyclic monoolefins in the range C_{22}—C_{30} separate micrococci into subgroups. These hydrocarbons are not found in staphylococci and can thus be used to differentiate between micrococci and staphylococci. Also, *Pseudomonas maltophilia* contains non-isoprenoid acyclic hydrocarbons, but they are different from those of micrococci and other pseudomonads. Acyclic isoprenoid hydrocarbons are more disseminated than non-isoprenoid hydrocarbons in bacteria. A number of bacteria contain squalene, but quantitative data are scarce.

Oxygenated tetracyclic triterpenes (sterols) have been isolated in *Methylococcus capsulatus*, where four major sterols have been described [92,93], and in trace amounts in other bacteria. Pentacyclic triterpenoid hydrocarbons have been identified in *Zymomonas mobilis* [94] and in *Bacillus acidocaldarius* [95].

As will be described under isoprenoids, naphthoquinones and benzoquinones may indicate phylogenetic differences between bacteria through their distribution patterns and structural variations [2]. Carotenoids also belong to isoprenoids. Oxygenated and non-oxygenated pigments of the C_{40} series and their precursors are widely distributed in bacteria.

Fatty acids are the lipid components most frequently used in chemotaxonomic studies on eubacteria [11]. Such acids constitute the lipids of the structural plasma membrane of Gram-negative outer membrane amphipathic polar lipids and are important parts of lipid A in lipopolysaccharides [4]. Both volatile and total fatty acids have been found useful in the characterization of bacteria, particularly non-hydroxylated fatty acids with up to 20 carbon atoms, and in some instances the fatty acid pattern is characteristic of a taxon [96]. In eubacteria, fatty acids are usually acylated to glycerol, sugar or amino compounds, whereas in archaebacteria they tend to occur in small amounts and mostly as free fatty acids. Eubacteria can be differentiated rapidly from archaebacteria through the demonstration of acid-stable ether lipids in the former [74]. Most eubacteria can be assigned to one of two broad groups on the basis of their non-hydroxylated fatty acid components [4]. Fatty acids can have branched, even-numbered or odd-numbered carbon chains, or combinations of these. They also occur as hydroxy fatty acids, cyclopropane fatty acids and mixtures of saturated and unsaturated acids. Hydroxy fatty acids are usually bound to sugars and amino compounds, and require hydrolysis to be released. The number of carbon atoms in fatty acids most often varies between 12 and 20 in bacterial cells and many groups, genera and species may be differentiated on the basis of their fatty acid patterns.

Metabolic acids are usually short-chain fatty acids and are useful in the taxonomy of anaerobic bacteria. Fatty acids of iso- or anteiso-configuration are usually found in Gram-positive bacteria, and in a few Gram-negative organisms. Polyunsaturated fatty acids are rarely present in eubacteria, but are of major taxonomic importance in blue-green bacteria. Cyclopropane fatty acids dominate in Gram-negative bacteria, with a few Gram-positive exclusions [96].

Hydroxylated fatty acids are usually present in lipopolysaccharides from Gram-negative bacteria [97] and also in ornithine lipids. Mycolic acids are esterified in the bacterial cell to an arabinogalactan linked to murein [4], and are long-chain 3-hydroxy acids with a long alkyl branch in position 2, found in *Caseobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus*. Structure comparisons of the mycolic acids have given valuable criteria in the classification and identification of members of these taxa, and many techniques have been developed to distinguish between the various types [98]. Mycobacterial mycolic acids are the most complex (60—90 carbon atoms) of these acids. Mycolic acids, which vary widely in structure, are of value for differentiation both between genera and between species. Mycobacterial mycolic acids can be distinguished by means of TLC of whole-organism methanolysates [99]. It is likely that the outer membrane formed by mycolic acids provides a matrix for other complex lipids of chemotaxonomic value in mycobacteria, such as glycopeptidolipids, sulphoglycolipids, glycosylated phenolphthiocerol esters and waxes based on phthiocerols [99]. The more polar glycopeptidolipids, being serologically active and type specific, are particularly valuable as chemotaxonomic markers [4].

Lipid analyses in bacteria have recently been reviewed [6,36,46,74,99—102].

3.3.1. Sample preparation

The extraction of polar lipids from bacteria and analysis by TLC frequently yield diagnostically important patterns. A mixture of solvents with comparative polarity is required to disrupt the hydrogen bonding, electrostatic forces and interfacial tensions to free membrane-associated polar and non-polar lipids. Large amounts of material can be extracted by the Bligh and Dyer method [103], as modified by Kates et al. [104]. With this method moderate amounts of solvents are used, and the output of lipid is usually high, 95% or more. The procedure of Folch et al. [105], which has been modified by Ways and Hanahan [106], gives a recovery of 95—99%. However, glycolipids may be lost [107].

Fractionation of lipids into their various classes, neutral and polar, can be effected by solvent partitioning and LC fractionation [74]. Partitioning of the lipids in cold acetone is recommendable when the lipid extract contains large amounts of pigments, neutral lipids or polar lipids. Whereas the acetone-soluble fraction contains neutral lipids, such as glycerides, sterols, sterol esters, hydrocarbons and carotenoids, the acetone-insoluble material contains virtually all of the phospholipids and polar pigments. Glycolipids can be recovered from the acetone-soluble fraction, while polyglycosyl diglycerides and sulphatides emerge with phospholipids in their acetone-insoluble fraction. Silicic acid columns with organic solvents may separate total lipid mixtures into various

classes of polarities [74]. Lipid extracts that have a high content of glycolipids, phospholipids and glycophospholipids can best be fractionated on an ion-exchange column such as DEAE-cellulose [108]. TLC is efficient for separating lipid complexes. Deacylation of column-fractionated and TLC-isolated lipids may assist in the identification of acyl glycerides and alkyl ether glycerides [42]. In order to analyse polar lipids that are alkyl glycerol ethers, or those having sugars and/or amino compounds linked to lipids, a number of methods have been recommended [74].

In most work reported the total content of fatty acids has been considered. Fatty acids are analysed after saponification with alkali or after methanolysis with anhydrous methanol—hydrochloric acid. Free fatty acids can be extracted with hexane in a Soxhlet apparatus directly from freeze-dried bacteria [35]. Methods for the analysis of the fatty acid composition of bacterial cells have been described by Jantzen and Bryn [100], Brondz and co-workers [13, 35, 43, 44], Kroppenstedt [101] and others. Numerical analyses of total fatty acid profiles are assuming increasing importance as an aid in the identification of bacteria [109].

Carotenoids are usually extracted with acetone. It should be realized that carotenoids are very susceptible to light and oxidation. Several other extraction methods can also be used with carotenoids [110—112].

Other isoprenoids are also highly sensitive to photooxidative degradation, and exposure to extreme pH values should be avoided. Collins et al. [113] recommend direct extraction of isoprenoids with chloroform—methanol.

Ideally, bacterial cells should be extracted as fast as possible after harvesting. Extensive conservation of the material can lead to changes in the lipoidal nature owing to lipopolytic and other enzymes being released from the cells. If immediate preparation of cells is impossible, they should be frozen and kept under a nitrogen atmosphere or be freeze-dried. Freezing of cells may lead to degradation, and lipids may be exposed to cellular enzymes. Low temperatures cannot protect bacterial lipids against lipolytic enzymes. Degradation of phospholipids has occurred in frozen bacterial extracts [114].

The solubility of chemically pure lipids in organic solvents differs markedly from that of the same lipids in complex cellular compounds of bacteria. The association of proteins with lipids or other polar molecules may significantly affect the extraction process. High concentrations of certain lipids may influence the solubility of others. It is difficult to predict the behaviour of lipids during extraction. The extraction of lipids from biological membranes has been reviewed by Zahler and Niggli [115]. Most organic solvents, during the extraction of lipids, will extract other substances simultaneously. Pre-extraction of tissue with 0.25% acetic acid can liberate extracts of potential contaminants, and simultaneously deactivate lipolytic enzymes [116,117]. This procedure has not been used in microbiology, but with plants and mammalian tissue. A more acknowledged method for the removal of non-lipid contaminants from lipid extracts is washing by liquid—liquid partition chromatography using Sephadex G-25 [118,119].

3.3.2. Gas chromatography of fatty acids

The conditions of cultivation and the age of the culture may affect the

composition of fatty acids both quantitatively and qualitatively. Thus, the composition of fatty acids in LPS from *Salmonella* species may vary with the culture temperature used [120]. Results should be interpreted in the light of this knowledge.

At the outset of the history of GC, efforts were made to analyse free fatty acids [121]. The main problem to overcome was the requirement of liquid phases to operate at high temperatures, molecular association of acids in the vapour state and acid adsorption on the column (for a review, see ref. 33). Recent years have seen considerable improvements in GC, particularly permitting stabilization of the stationary phase at relatively high temperatures [122]. This has made it possible to separate fatty acids up to C_{20} by means of GC. One of the initial achievements was the separation of fatty acids on glass bead columns [32]. However, the separation of long-chain fatty acids and the symmetry of the peaks were not satisfactory. Considerable improvements to capillary columns with the development of temperature-resistant stationary phases led to satisfactory separations and symmetry of peaks from a mixture of synthetic C_6—C_{18} fatty acids [35] and bacterial fatty acids [13, 35, 123]. C_2—C_{10} acids were well resolved on a fused-silica capillary column with minimal peak tailing and adsorption [124]. After publication of our method for the separation of free fatty acids [35], the separation of C_8—C_{24} free fatty acids on a capillary column was reported [125] and claimed as a new method. In all these investigations [35, 123—125] both saturated and unsaturated acids were well resolved. It was also possible to distinguish between fatty acid isomers, i.e., $C_{15:0}$, iso-$C_{15:0}$ and anteiso-$C_{15:0}$ [123]. It therefore seems justified to recommend the use of capillary columns for the GC separation of both saturated and unsaturated fatty acids, including branched-chain fatty acids, from C_2 to C_{24}. The direct analysis of fatty acids makes it unnecessary to resort to complicated derivatization techniques [126—128], which tend to lengthen the overall analytical procedure, and preclude the non-quantitative recovery of esters from lower acids [127].

Derivatization techniques for GC analyses of fatty acids have been well reviewed [90,129]. The most commonly used methods include acid-catalysed esterification, methylation with diazomethane, boron trifluoride or boron chloride in methanol and methylation with hydrochloric acid in methanol. None of these techniques exclude the possibility of artifacts being formed from a naturally existing lipid mixture. Many workers consider that hydrochloric acid in anhydrous methanol is a mild and useful derivatization reagent for lipids. For a complex mixture consisting of saturated, unsaturated, hydroxy, cyclopropane and other fatty acids, it may be difficult to provide an efficient derivatization procedure that does not produce artifacts. Thus, base- or acid-catalysed methylation may produce unsaturated artifacts from hydroxy acids [130]. Alkaline esterification is preferred to acid methanolysis when the material contains cyclopropane fatty acids, as it will not attack cyclopropane rings [131]. Low recoveries of hydroxy fatty acids can be expressed by using methanol—sulphuric acid [132]. Artifacts produced during methylation with boron trihalides have also been reported [133]. Diazomethane methylation, as modified by Schlenk and Gellerman [134], gives few by-products and under proper conditions it is moderately dangerous to health despite the high carcino-

genicity and other negative aspects of diazomethane reagents [11]. Recently, Bauer et al. [135] found that derivatization with diazomethane also is not free from artifacts. These were characterized by GC—MS [135]. Trifluoroacetates may attack methylene groups between double bonds [136] or cyclopropane fatty acids. Trimethylsilyl derivatives cause isomerization of partial glycerides [137, 138] and are easily hydrolysed. *tert.*-Butyldimethylsilyl (t-BDMS) derivatives are more stable during hydrolysis [139]. The GC analysis of hydroxy fatty acids may be difficult to perform. If the hydroxy group is not derivatized, the result may be adsorption of acid on the column and non-symmetric peaks [90]. Satisfactory results were obtained in the GC analysis of methyl esters of hydroxy acids without derivatization of the hydroxy group [140]. The adsorption, which is irreversible owing to transesterification with the support material, causes incomplete recovery of the acid. The hydroxy group should therefore be derivatized before GC analysis. A number of derivatization methods exist for the GC analysis of hydroxy fatty acids [141—145]. Acetylation with acetic anhydride in pyridine has been described [142]. Acetyl methanesulphonate in microcolumns of Celite can be used for routine analysis [143]. Another method is acetylation at high temperature with acetic anhydride [144]. Trifluoroacetylation with trifluoroacetic anhydride in pyridine provides high temperature-stable derivatives [136] and is often used in lipid analyses (e.g., refs. 73 and 75). TMS derivatives, and trialkylsilyl ether derivatives other than TMS, have been used extensively for the analysis of fatty acids with hydroxy groups, and their preparation and properties have been reviewed [146—149]. Derivatives of t-BDMS, which are considerably more stable than TMS derivatives, have also been recommended [139]. Isopropylidene compounds have been used for the GC analysis of vicinal diol-dihydroxy acids [150], and *n*-butyl boronates can be used for studying the structure of 1,2- and 1,3-diols of α- and β-hydroxy acids as they form five- and six-membered rings, respectively [151, 152].

3.3.3. Mass spectrometry of fatty acids

The mass fragmentation of fatty acids has been studied mainly on the basis of methyl esters [153—157]. Such esters usually give measurable M^+ and characteristic fragments, i.e., M−32 (loss of methanol) and the "McLafferty rearrangement" ion [155]. Pyrrolidide derivatives of fatty acids provide measurable M^+ and their fragmentation has been described [158]. Other derivatives suitable for MS include quaternary ammonium salts. The position of the double bonds in fatty acids has been studied by oxidation to vicinal diols, which were subsequently derivatized and analysed in the form of isopropylidene [159] or TMS ether derivatives [160—164]. Mercury acetate adducts have been used for the MS examination of the positions of double bonds [165—167]. Dimethyl disulphide adducts are also convenient derivatives for this purpose. The positions of triple bonds can be determined in pyrrolidides from fatty acids without specific derivatization of the triple bonding [168]. Cyclopropane methyl esters from fatty acids are converted into suitable derivatives before examination by MS [157, 169—171]. Pyrrolidides or methyl esters of cyclopentene, cyclohexane and furanoid fatty acids may be identified by MS [172—175]. The positions of α- and β-hydroxy groups in

methyl esters have been studied by MS [176]. Branching positions in fatty acids are not easy to establish by MS without transforming the acids. One of the most commonly used procedures is oxidation by means of an acid and potassium permanganate. The MS of free fatty acids has been relatively little used in the determination of natural fatty acids. Fatty acids give M^+ of approximately 12% in electron impact MS and a series of characteristic ions, e.g., *m/e* 60, M–28 [123]. The abundance of the *m/e* 60 "McLafferty rearrangement" ion in saturated free fatty acids is approximately 62%, in branched acids approximately 50% and in monounsaturated acids approximately 20% of the base peak. These relative abundances may be used diagnostically [13, 123].

3.3.4. High-performance liquid chromatography

Fatty acids have no suitable chromophore for the detection of visible, ultraviolet or fluorescent light and it is therefore difficult to analyse fatty acids by means of HPLC. Refractive index detection has been used most commonly when underivatized lipids or fatty acids have been analysed by means of HPLC. The refractive index detector has low stability and sensitivity. A variable-wavelength UV—visible detector has often been used in the range 195—206 nm. The carboxylate functional group of fatty acids is a weak chromophore ($\lambda_{max.}$ = 204 nm, log ϵ = 1.78). The few solvents applicable as a mobile phase are those which are transparent in this range (195—210 nm). Relatively well established methods are available for the analysis of fatty acids by GC and the impetus to analyse fatty acids by HPLC has therefore not been large. In addition analyses of fatty acids by HPLC have until recently not been quantitative. A limited number of studies have been made in which underivatized fatty acids of microbial origin have been analysed by HPLC (e.g., ref. 177). The analysis of bacterial metabolites using a cation-exchange resin column and a UV detector has been carried out at 220 nm [178] and 210 nm [177, 179, 180]. *Clostridium* cultures and a group of other clinically important bacteria have also been examined by this technique [177, 180].

From the outset of fatty acid analyses by HPLC, efforts were made to improve the sensitivity of detection and separation by derivatization [181, 182]. Derivatives have been separated on reversed-phase columns. The rapid preparation of phenacyl and naphthacyl derivatives [181] and *p*-bromophenacetyl esters via crown ether-catalysed reactions [183] has been described. The preparation and separation of pentafluorobenzyl esters with detection at 254 nm [184] and derivatization of carboxylic acids by reaction with 4′-bromophenacetyl trifluoromethanesulphonate with detection at 254 nm [185] have also been reported.

A number of procedures have been described for the detection of fluorescent derivatives in extremely low concentrations with a fluorescence detector [186]. Derivatization reagents used for this purpose include 9-anthryldiazomethane [186], 4-bromomethyl-6,7-dimethoxycoumarin [187], 9-(hydroxymethyl)anthracene [188] and 9-aminophenanthrene [189]. Derivatives for visible range (400 nm) detection at concentrations up to 10—15 pmol have been described [190].

GC rather than HPLC is preferred for the analysis of bacterial fatty acids as methyl esters. In routine work the methyl esters of fatty acids are most often used.

3.3.5. Thin-layer chromatography

Methyl esters of fatty acids can also be separated by TLC and this technique has made an important contribution to the separation and isolation of lipids. Silver ion TLC is the most commonly used method for the separation of unsaturated fatty acids [191,192] and *trans*- and *cis*-isomers of fatty acids can also be separated easily [191,193]. The separation of fatty acids by two-dimensional TLC can also be performed [194]. The long-standing problem with the quantification of components has now been solved through the development of photometric detectors for TLC. Progress in photometric methods of quantification in TLC has been reviewed [195]. However, like HPLC, TLC cannot compete with GC in the analysis of fatty acids from bacteria.

3.4. Lipopolysaccharide

Lipopolysaccharide (LPS) is a macromolecule that may give valuable information both on the taxonomic position of a bacterial strain and on its phylogenetic relationships [60]. Such information may be derived not necessarily from full analysis of the molecule, but through analysis of its main constituents, the O-specific part, the core or lipid A. Core-specific sugars, amide-linked fatty acids or unusual backbone sugars in this macromolecule may all provide valuable taxonomic information. This may also be achieved by analyses restricted to components that rarely occur in LPS (Fig. 4).

In the O-specific chain, the sugar constituents, their sequences and their modes of linkages are important for the serological and chemical specificity of the strains and thereby of taxonomic significance. In *Salmonella*, *Escherichia*, *Citrobacter*, *Proteus*, *Xanthomonas* and others, sugar constituents of LPS are the main determinants for the classification of serotypes. A number of sugars have been identified in LPS, including neutral sugars, branched-chain sugars, O-methyl sugars, amino sugars and acidic sugars. Specific sugar components of unusual structure, often in a terminal position, can serve as immunodominant sugars in many O-specific groups. The O-specific chain may also be absent, or very short, consisting of only a few sugar residues. In *Rhodopseudomonas gelatinosa*, *Neisseria* and *Yersinia pseudotuberculosis* ssp. *pestis* it is completely missing [196—198].

Important taxonomic information may be obtained from identification of the core, which is a short acidic hetero-oligosaccharide of repeating units. Incomplete core structures of *Salmonella* mutants have been designated Rb, Rc, Rd and Re. Mutants of *Salmonella*, defective in the biosynthesis of their O-antigens, can also be characterized by R-specific phages [199]. Whereas *Salmonella* serotypes have the same core structure, *Escherichia coli* has five serotypes with different complete R-core structures (R1—4 and K-12) [199—201]. All have a different outer (hexose) region, whereas the inner region is constant. *Shigella* has the same R-core as *Escherichia*. R-specific phages and precipitation with lectins have occasionally been used to differentiate between core structures [202]. Also in *Proteus mirabilis* R mutans there are different core types, as indicated by the presence of galactosamine and the rare sugar D-glycero-D-mannoheptose, in addition to the more common L-glycero-D-mannoheptose [203]. *Yersinia pestis* and *Actinobacillus actinomycetemcomitans*

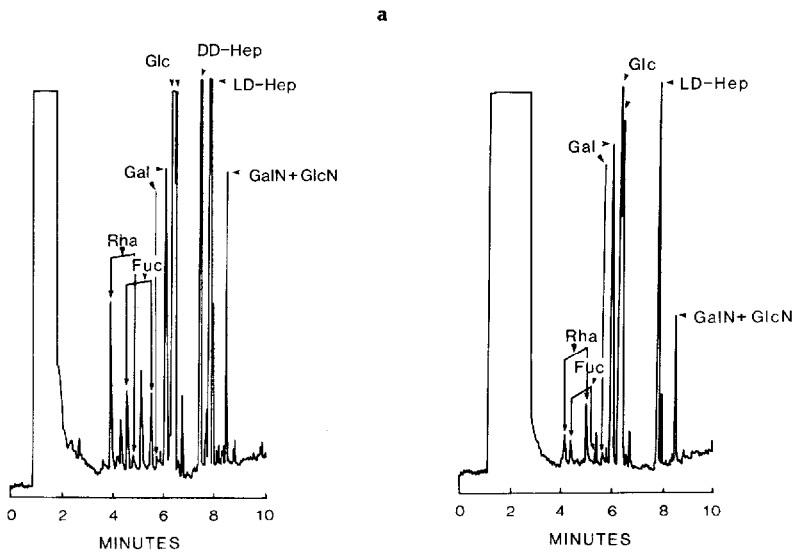

Fig. 4. Typical gas chromatograms of the sugar composition in lipopolysaccharide from (a) *Actinobacillus actinomycetemcomitans*, as represented by strain ATCC 33384, and (b) *Haemophilus aphrophilus*, as represented by strain ATCC 33389. Abbreviations: Rha = rhamnose; Fuc = fucose; Gal = galactose; Glc = glucose; DD-Hep = D-glycero-D-manno-heptose; LD-Hep = L-glycero-D-mannoheptose; GalN = galactosamine; GlcN = glucosamine. (Courtesy J. Chromatogr.)

also have a core with L- and D-glycero-D-mannoheptose [45,204]. Certain bacterial species lack heptose and/or 2-keto-3-deoxy-octonate (KDO) [205]. Thus, heptose has not been detected in LPS from *Xanthomonas*, *Myxobacteria*, *Moraxella*, *Branhamella* and *Rhodospirillaceae* spp. [206]. *Bacteroides*, *Leptotrichia* and *Vibrio cholera* seem to lack KDO. Substituted or chain-linked KDO may avoid detection during analysis. KDO and heptose may also occur in polymers other than LPS.

Lipid A is the most conservative part of LPS. Free lipid A in *Salmonella* consists of β-1,6-linked D-glucosamine disaccharide carrying phosphate residues in positions 1 and 4, each differently linked to glucosamine. The backbone of lipid A is substituted by 4 molecules of (*R*)-3-hydroxy fatty acids, two of them being amide linked and the other two ester linked to positions 3 and 3′ [207]. In free lipid A, the substitution of 3-hydroxy fatty acids by additional acids may vary between species. The fatty acid linked to the amino group of GlcN II can be substituted by $C_{12:0}$, $C_{14:0}$ or $C_{16:1}$ acids [120,208]. The nature of the substituents of the two phosphate groups attached to the lipid A backbone is often characteristic of different species, but the degree of substitution may vary between the strains owing to the different culture conditions used. A number of bacteria have similar backbone structures in lipid A: *Enterobacteriaceae*, *Pseudomonadaceae*, *Fusobacterium nucleatum*, *Selenomonas ruminantium* [205] and some *Rhodospirillaceae* [209]. Lipid A types (I—IV) of *Rhodospirillaceae* correspond with 16S rRNA homology studies [210,211]. Interestingly, the different lipid A groups differ in toxicity. Variations in the fatty acid substituents of lipid A may be of taxonomic value [205, 212, 213]. The chain length of amide-linked fatty acids generally varies between $C_{10:0}$ and $C_{16:0}$,

depending on the bacterial species or other taxonomic units [60]. 3-OH-$C_{14:0}$ is present in *Enterobacteriaceae*, 3-OH-$C_{12:0}$ in *Pseudomonadaceae* and 3-OH-$C_{16:0}$ in *Rhizobiaceae* and the *Rhodospirillaceae* group III. A number of families or phylogenetic clusters of bacterial species show a combination of characteristic amide- and ester-linked fatty acids that can serve as taxonomic markers [213]. It should be realized that 3-hydroxy fatty acids also are present in polar lipids such as ornithine-containing lipids [214]. Methods recommended for extraction of LPS and for the determination of fatty acids and sugars in LPS have recently been reviewed [60].

For extraction of LPS, it is most common to use the phenol—water method [215]. Extracts recovered by this technique have to be purified. Galanos et al. [216] introduced extraction with phenol—choloroform—light petroleum, with which it is possible to achieve the quantitative extraction of R-type LPS of high purity. Extraction of LPS with dimethyl sulphoxide has also been proposed [217]. This procedure yields extracts that are highly contaminated with proteins. Both S- and R-type LPS of high purity can be obtained by using the method of Darveau and Hancock [218], but this procedure is labour intensive. Diethylene glycol selectively extracts free polysaccharides and the O-antigen complex [219]. Extraction of LPS with trichloroacetic acid was introduced by Boivin and Mesrobeanu [220]. With this method a highly immunogenic LPS—protein—phospholipid complex is obtained.

3.5. Murein

Detailed chemical information on murein has been given [72]. Whereas murein (peptidoglycan, mucopeptide) is a typical stable cell wall polymer in eubacteria, it is absent from archaebacteria, L-forms of bacteria and mycoplasmas. Actually, murein is a key factor in the separation of eubacteria from archaebacteria. A pseudomurein, characterized by the muramic acid being replaced by talosaminouronic acid, is the typical cell wall constituent of the genus *Methanobacterium*, which belongs to the archaebacteria [221].

In Gram-positive bacteria, murein is a major cell wall component, constituting over 30% of the dry weight of the wall. Here, murein is covalently linked to protein, polysaccharide and/or teichoic or teichuronic acid. In Gram-negative bacteria, murein usually constitutes less than 10% of the dry weight of walls and is covalently linked to lipoprotein.

Murein is a heteropolymer that consists of polysaccharide strands (glycan) cross-linked through short tetrapeptides [222]. The glycan consists of polymers of N-acetylglucosamine and N-acetylmuramic acid. The mono sugars are pyranose-formed and β-(1,4)-glycosidically linked to each other. A so-called stem peptide (oligopeptide), consisting of alternating L- and D-amino acid, is connected with the carboxyl group of muramic acid. Adjacent stem peptides can also be cross-linked directly or via interpeptide bridges. Murein is a relatively large molecule with an approximate molecular weight of $1 \cdot 10^7$, which encompasses the entire bacterial cell. In bacteria, the peptide moiety of murein varies considerably, whereas the glycan portion is fairly uniform. Variation in the glycan moiety has been reported in bacilli [223] and in rhodopseudomonads [224], which tend to possess no N-acetyl substitution of glucosamine,

and in *Micrococcus luteus*, where there is no acetylation of the amino group in muramic acid [225]. Muramic acid is oxidized to give an N-glycolyl residue in some mycobacteriae and nocardiae, and may be a valuable chemotaxonomic marker [226,227]. Substitution of N-acetylmuramic acid by N-glycolmuramic acid in some mycolic acid-containing actinomycetes may be of taxonomic value [228].

As for the stem peptide, almost 100 different chemical variations have been demonstrated in bacteria. There is always an alternation of L- and D-amino acids in the stem peptide. Also *meso*-diaminopimelic acid is bound with its L-asymmetric centre to the stem peptide. The amino acid sequence of the stem peptide varies between different mureins. Diamino acids in the peptidoglycan of actinomycetes and coryneform bacteria are major taxonomic characters [4]. Probably organisms containing different diamino acids should not be classified in the same genus. There is also variation in the cross-linking of stem peptides, through which two major mureins can be formed, A and B [222]. The amino acid sequence of the stem peptide varies among different mureins. Group A murein is much more widely distributed in bacteria than group B murein, which is found only in some coryneform and in a few anaerobic bacteria [229]. A characteristic feature of murein in Gram-negative bacteria is that, with a few exceptions, it is cross-linked directly and contains *meso*-diaminopimelic acid as the diamino acid in position 3 of the stem peptide [222]. *meso*-Diaminopimelic acid is replaced by ornithine in *Spirochaeta stenostrepta* and *Treponema pallidum*, by *m*-lanthionine in some fusobacteria and by lysine in *Bacteroides assaccharolyticus*.

Murein is a valuable taxonomic marker especially in Gram-positive bacteria, where it is multi-layered and occurs with great variations [72]. There are two important aspects to consider here: the chemical composition and the structure of murein. Qualitative amino acid analyses of cell walls have provided taxonomic information in certain propionibacteria, cocci of the *Streptococcus mutans* group and in the differentiation of oerskoviae, certain micrococci and bifidobacteria (for a review, see ref. 222). Amino acid analyses have also permitted the separation of species of *Enterococcus faecalis* from other enterococci and of staphylococci from micrococci. Glycolyl residues in the cell walls of certain coryneform bacteria may also be used as taxonomic markers [230]. The amino acid compositions of the cell wall from bacteria can be identical, although the murein type can differ. Determination of the murein type has been described [222, 229]. Murein structures are particularly important in the taxonomy of Gram-positive bacteria. Murein structures show good correlation with lipid and DNA base composition. However, the presence of the same murein structure does not necessarily imply that bacteria are closely related.

3.5.1. Isolation of cell walls and murein from Gram-positive bacteria

After harvesting of cells, cell enzymes are deactivated and, if necessary, cells are defatted [231,232]. Thereafter, destruction of cells is performed, usually by mechanical methods. The suspension of destroyed cells is centrifuged at 30 000—40 000 rpm for 20 min. The sediments consist of cell walls contaminated with other substances. Further purification of the cell

walls is performed by trypsination of the crude substance. After treatment with trypsin, relatively pure cell walls are obtained, which may contain polysaccharides or teichoic acid polymers. To remove DNA or RNA polymeric contaminants from the cell wall preparations, DNAase and RNAase are used. For the removal of fragments of other bacterial membranes from cell wall preparations, extraction with sodium dodecyl sulphate is performed [233]. Pure murein can be obtained by the method of Schleifer [234]. *Neisseria gonorrhoeae* murein was analysed by reversed-phase HPLC by Dougherty [235], who described murein preparation, fractionation and amino acid analysis. Amino sugar analyses in streptococcal cells have also been reported [236]. Extensive details of murein analysis were given in ref. 237.

3.5.2. Isolation of cell walls and murein from Gram-negative bacteria

Weidel et al. [238] described a procedure for the extraction of murein from *Escherichia coli*. Murein has also been prepared from other Gram-negative cell walls [239—241]. Analysis of murein from Gram-negative bacteria is similar to that from Gram-positive species (Section 3.5.1).

3.6. Cellular proteins

The microbial genome results in the synthesis of about 2000 protein molecules that constitute the microbial cell. These molecules represent an information source of immense potential for the characterization of microorganisms [242]. The protein composition of microbial cells reflects their genetic capacity, and closely related organisms will have similar or identical kinds of cellular proteins. These may be depicted from organisms through fingerprints on electropherograms. The application of whole-cell protein PAGE electrophoresis in the microbial classification, differentiation and identification of a great variety of bacteria has been reviewed [243,244]. The results parallelled those from DNA—DNA hybridization in several genera, including *Agrobacterium*, *Brucella*, *Propionibacterium*, *Pseudomonas* and *Zymomonas* [242]. Different species and subspecific taxa usually exhibit great variations in their protein patterns. *Zymomonas* seems to have a genus-specific protein electropherogram [245]. Soluble and cell-envelope protein similarities can be detected within species, subspecies, biotypes or strains. Visual or computer-assisted comparisons of electrophoretic protein patterns have several advantages, and a few drawbacks [243].

Electrophoresis of bacterial proteins is usually carried out after soluble proteins have been moved in a polyacrylamide gel, or the native proteins of a bacterial strain are submitted to electrophoresis in a stabilizing medium and stained for certain enzymes [246]. One-dimensional protein electophoresis has a much lower resolution than two-dimensional electrophoresis, where up to 1200 protein spots can be detected [247]. The one-dimensional technique is also inadequate in cases of pleiotrophic effectors, developing transitions or mutations [247]. By means of high-resolution, two-dimensional protein electrophoresis it was possible to distinguish between the closely related *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* and even to delimit strains within both species [248]. This had been difficult to achieve

by GC. Two-dimensional separations of total cellular proteins, which have also been used for comparison of *Rhizobrium* [249] and *Spiroplasma* strains [250], may lead to more accurate classification and identification owing to the exceedingly detailed and specific fingerprint system it provides and it appears to have a high potential in chemotaxonomy. Thus, in the protein patterns from the B and K12 strains of *Escherichia coli* there was a difference in the position of almost 20% in the proteins resolved [251].

In contrast to whole-cell proteins, ribosomes of procaryotes are made up of a limited number of proteins that have more limited variability. These proteins may aid in the identification of an isolate, or in the tentative classification, of closely or moderately related bacteria. Immunological comparisons of the

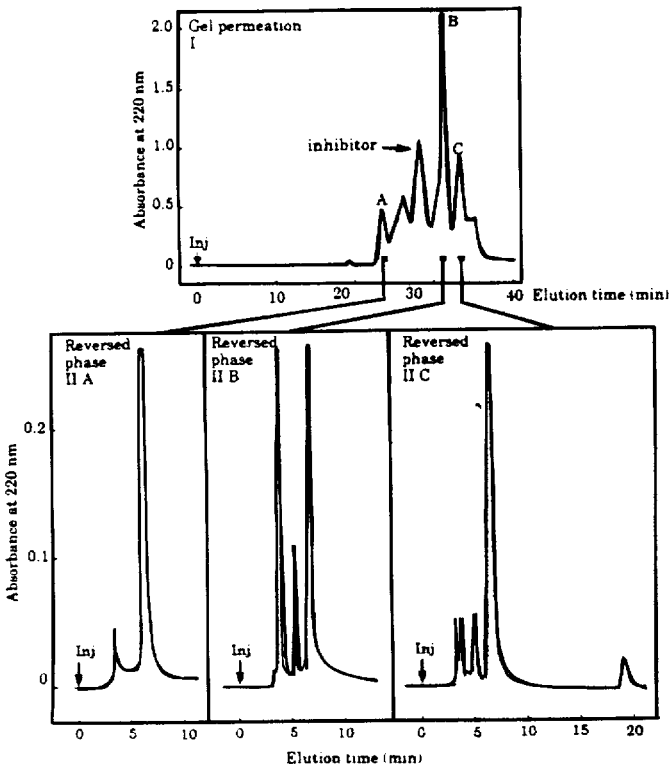

Fig. 5. Separation of enzymic digests by high-performance reversed-phase chromatography after gel permeation chromatography. Sample: tryptic digest of protein A (50 min at room temperature). I, 0.9 mg; IIA, 30 μg; IIB, 35 μg; IIC, 35 μg. The indicated fractions from I were dried, then dissolved in mobile phase II before injection. Injection volume: I, 200 μl; II, 20 μl. Fraction volume: 4 drops (200 μl). Temperature: 22°C. Pressure: I, 40 bar; II, 70 bar. Mobile phase I (GPC): 0.1 *M* Na_2HPO_4—NaH_2PO_4, pH 6.5. Mobile phase II (RPC): 0.1 *M* NaH_2PO_4*, 0.1% triethylamine**, H_3PO_4 to pH 3.0, 25% (v/v) acetonitrile. Flow-rate: I, 500 μl/min; II, 600 μl/min. Detection: 220 nm; I, 2.56 a.u.f.s.; II, 0.32 a.u.f.s. *Phosphate used as hydrophilic ion pairing agent. **Capping agent (for residual silanol groups). (Courtesy LKB Produkter AB, Bromma, Sweden.)

separated proteins can be made [252]. Analysis of ribosomal proteins by two-dimensional gel electrophoresis has been found useful for a number of bacteria, e.g., *Enterobacteriaceae* and *Bacillaceae* [252]. It has also proved valuable in the analysis of organisms for which identification criteria are not available, e.g., methanogenic bacteria. Protein A is a cell wall constituent of *Staphylococcus aureus* which exhibits a strong binding affinity to the Fc region of immunoglobulin G. Owing to a repeating unit structure in this protein of molecular weight 42 000, a partial tryptic cleavage will generate mono-, di-, tri- and tetramers of a peptide unit of molecular weight 7000. Several low-molecular-weight fragments will also arise, as shown in Fig. 5.

3.6.1. Cytochromes

Cytochromes are involved in a number of redox processes of the procaryote cell, such as photosynthetic electron transfer and chemolithotropic, heterotrophic and anaerobic respiration [4]. Cytochromes are specialized haemoproteins consisting of a haem prosthetic group tightly bound to a protein. In haem, four pyrrole rings are joined by methenyl bridges to form porphyrin [253,254]. The central iron atom of porphyrin oscillates between the reduced and oxidized states. The C-2, C-4, C-5 and C-8 positions at the periphery of the haem molecule can be substituted, and these substituents are largely responsible for different absorbance properties and redox potentials of various cytochromes. Cytochromes can be assigned to four major classes, a, b, c and d, according to the structure of the haem prosthetic groups. Cytochrome o is an auto-oxidizable b type cytochrome [255]. Cytochrome patterns are used as increasingly powerful tools in bacterial classification [253,254]. Cytochrome structures have also been examined, but these analyses are restricted to cytochrome c. Cytochrome patterns alone cannot be used for identification, because bacteria contain relatively few types of spectrally distinct cytochromes [255].

For easier compilation and interpretation of cytochrome results, Jones and Poole [254] subdivided bacteria into several broad groupings on the basis of their metabolic behaviour and/or reaction to Gram stain: (1) chemoheterotrophs (Gram-positive and Gram-negative eubacteria, archaebacteria), (2) chemolithotrophs (Gram-negative eubacteria, archaebacteria and (3) phototrophs (Gram-negative eubacteria).

Gram-positive chemoheterotrophs (*Micrococcaceae*, *Actinoplanaceae*, *Streptomycetaceae*, most *Bacillaceae* and other families) usually have cytochrome patterns of the $bcaa_3o$ type. Some variations exist. Thus, a few species contain only one cytochrome oxidase, and cytochrome c is often absent. Gram-negative chemoheterotrophs (*Azobacteriaceae*, *Vibrionaceae*, *Enterobacteriaceae*, *Pseudomonadaceae* and others) exhibit much less homogeneous patterns during aerobic growth. Most of these organisms have a cytochrome $bcoa_1d$ pattern where cytochrome c often is absent. While these organisms do not synthesize cytochrome oxidase aa_3, some of the organisms of this major group do, such as species of *Pseudomonas* and most methylotrophs, *P. denitrificans*, genera of *Halobacterium* and *Rhizobium*, thereby exhibiting the cytochrome $bcaa_3o$ pattern. *P. fluorescens*, *P. cichorri* and *P. aptata*, which do not synthesize a- or d-type cytochromes, have simple bco or bo patterns.

Chemolithotrophs (*Nitrobacteraceae*, *Thiobacillaceae*, species of *Pseudomonas* and others) generally have cytochrome patterns of the $bcaa_3o$ type; cytochrome c is usually present. Denitrifying chemolithotrophs contain cytochrome cd_1.

In the phototrophs (*Rhodospirillaceae*, *Chromatiaceae*, *Chlorobiaceae*, *Chloroflexaceae* and *Cyanobacteriaceae*), b and c cytochromes are found. Neither phototrophs nor chemolithotrophs produce cytochrome d [255].

Analyses of cytochrome structures are usually concerned with the primary structures and, when possible, the tertiary structure of easily purified cytochrome c. Structures are determined by analysis of amino acid sequences and X-ray diffraction patterns [256—258]. Most of these are type I cytochrome c, as detected in *Rhodospirillum* or *Rhodopseudomonas*, from the obligate phototroph *Chlorobrium* and from a wide range of chemoheterotrophs such as *P. denitrificans*, *A. vinelandii*, *Micrococcus* spp. and several pseudomonads. Type I cytochromes can be divided further into three groups according to the number of amino acids present: long, medium and short. Examination of cytochrome structures has indicated that Gram-negative chemoheterotrophs are closely related to certain phototrophs, probably having evolved from photosynthetic ancestors through loss of the light-harvesting apparatus [254].

The single most useful analytical technique for cytochromes in bacteria has been spectrometry, which provides information on the identity, quantitation and functional properties of the cytochromes, as obtained from intact cells, subcellular fractions or purified or partially purified preparations [254].

Spectrometry may also contribute to the structural determination of the haemoprotein structure, although NMR, vibrational and Mössbauer spectroscopy, circular dichroism and magnetic susceptometry are gaining increasing importance here [259]. Non-spectrophotometric techniques have also been found useful for the analysis of cytochromes [253,254]. Both the quantitative and, to a lesser extent, the qualitative cytochrome contents of bacteria are influenced by their growth conditions [255].

3.6.2. Enzymes

The structural and functional patterns of certain bacterial enzymes have been used to assist in bacterial classification. Examples are the regulatory and molecular size patterns of bacterial citrate synthases and succinate thiokinases [255], both of which are enzymes of the citric acid cycle and of universal occurrence. Citrate synthases of Gram-negative bacteria, but not those of Gram-positive bacteria, are reduced by NADH. The citrate synthases of strictly aerobic Gram-negative bacteria are reactivated by AMP whereas those of facultatively anaerobic bacteria are not. α-Oxoglutarate inhibits citrate synthases of Gram-negative facultative anaerobes, whereas the corresponding enzymes from the aerobic Gram-negative bacteria are not inhibited. Citrate synthases of cyanobacteria are inhibited by α-oxoglutarate and succinyl-CoA, but not by NADH.

Bacterial citrate synthases may also be differentiated according to molecular size. Most Gram-negative bacteria have large citrate synthases (molecular weight = 250 000), whereas Gram-positive bacteria usually produce small citrate synthases (molecular weight = 100 000). There are exceptions to this rule.

Bacterial succinate thiokinases show the same molecular size patterns as citrate synthases. Succinate thiokinases from Gram-positive bacteria are of the small type (molecular weight = 70 000—75 000), whereas those of Gram-negative bacteria, cyanobacteria and *Halobacterium* species are of the large type (molecular weight = 140 000—150 000). Further subdivision of bacterial succinate thiokinases may be made according to the specificity for nucleotide substrates.

A method based on the ability of cellular hydrogenases to reduce the redox indicator methylene blue was recently devised to assist in the differentiation between closely related species within *Actinobacillus*, *Haemophilus*, *Pasteurella*, *Streptococcus* and *Bacteroides* [260].

Replacement of conventional tests by measurement of enzyme activities may reduce the time and labour involved in the characterization of bacteria [261]. The application of such techniques to urine specimens could permit the identification of pathogens in 1—2 h.

Electrophoretic enzyme patterns may be valuable when applied with caution to taxa that can be clustered by other criteria [246]. The patterns of enzyme mobility may vary amongst strains of species, or between subspecies or species. However, identical patterns may also be obtained with different species or with different genera.

Little attention has been devoted to enzymes of non-bacterial origin in chemotaxonomy, despite the fact that such enzymes are often used by various hosts to counteract bacterial propagation. With hen egg white lysozyme and EDTA, it was recently possible to differentiate between closely related species of the *Actinobacillus—Haemophilus—Pasteurella* group [13, 262].

3.6.3. Amino acid sequences of proteins

Amino acid sequences of specific bacterial proteins have been used as an indication of the phylogenetic relationship between microorganisms, and in many Gram-positive taxa it has led to major revisions. It is likely that most proteins have developed from a relatively small number of archetypical proteins by genetic duplication and modification [263]. Large differences in the amino acid sequence of bacterial proteins should therefore indicate major differences in the evolution of these organisms. Phylogenetic schemes have been developed over the years, reflecting the presumed evolutionary development of many organisms, both prokaryotes and eukaryotes. The proteins most often examined include ferrodoxins, flavodoxins, azurins, plastocyanins and cytochrome c [255]. Ambler [264] has conducted extensive sequence studies on bacterial proteins. Such studies will probably assume increasing importance in chemotaxonomy. It should be realized that comparison of amino acid sequences has certain limitations [263]. Analysis of cell wall amino acids by GC is a fast and convenient method supporting the identification of many Gram-positive bacteria. HPLC is a highly sensitive method for the analysis of phenylthiohydantoin derivatives. The application of conventional and microbore chromatography has been discussed by Cunico et al. [265] and previously by Deyl [266], who reported that phenylthiohydantoin derivatives have great potential in amino acid analysis. Rapid analyses of amino acids using pre-column derivatization have been described [267]. The extraction of amino

acids or peptides from bacteria may result in complex mixtures of amino acids, dipeptides and oligopeptides, which must be separated before further analysis is undertaken. Giliberti and Niederwieser [268] described the separation of neutral oligopeptides, neutral dipeptides and amino acids as copper complexes. The procedure involves the separation of amino acids from peptides on DEAE-Sephadex A-25 at pH 8.0 and further separation of oligopeptides from dipeptides on DEAE-Sephadex A-50. The separation is based on differences in the charges of copper complexes of amino acids, dipeptides and oligopeptides. Sasagawa et al. [42] described the separation of peptides on a polystyrene resin column, and Otsuka et al. [269] detailed the electrokinetic chromatographic separation of a mixture of 22 phenylthiohydantoin amino acids. An example of two-dimensional TLC studies on amino acids is the work of Keller et al. [270].

Soluble enzymes can be precipitated by adding ethanol or acetone. The mixture should be kept at 0°C to reduce denaturation. Precipitation with inorganic salts, e.g., ammonium sulphate, sodium sulphate or potassium sulphate, is also a valuable method.

3.7. Isoprenoid quinones and carotenoids

Isoprenoid quinones, which are a class of terpenoid lipids, are located in the cytoplasmic membranes of most procaryotes. Here they play important roles in electron transport, oxidative phosphorylation and active transport. Their taxonomic importance has been realized only recently. Naphthoquinones and benzoquinones are the two most important structural groups of bacterial isoprenoid quinones [102]. Naphthoquinones can be further separated into phylloquinones and menaquinones. Phylloquinones have so far only been detected in cyanobacteria as a 2-methyl-3-phytyl-1,4-naphthoquinone. Naturally occurring menaquinones contain a large class of molecules where the C-3 multiprenyl side-chain varies from 1 to 15 isoprene units [102]. Varying degrees of saturation or hydrogenation of the C-3 multiprenyl side chain also occur in menaquinones. Dihydrogenated menaquinones are frequently found in corynebacteria and mycobacteria. More highly saturated menaquinones have been seen in certain actinomycetes [271]. The methyl group of menaquinone at C-2 may also be replaced with a hydrogen atom (demethylmenaquinone) [271]. The latter has been found in many Gram-negative eubacteria, rarely in Gram-positive species, but not in archaebacteria. Chlorobiumquinone, isolated from green photosynthetic bacteria, is the only bacterial isoprenoid quinone that contains a side-chain carbonyl group. Interesting new quinones recently demonstrated in bacteria include methionaquinone, thermoplasmaquinone and methyl-substituted menaquinone-6 [102].

The bacterial isoprenoid benzoquinones contain two major classes: ubiquinones and plastoquinones. Ubiquinones are chemically a 2,3-dimethoxy-5-methyl-1,4-benzoquinone nucleus with a multiprenyl side-chain in position 6. The side-chain varies from 1 to 15 isoprene units. In contrast to menaquinone, ubiquinones with partially saturated side-chains are not found in bacteria. Ubiquinones have not been detected in Gram-positive eubacteria or archaebacteria, only in Gram-negative eubacteria [102]. Plastoquinone, which is the

other major bacterial quinone, is restricted to cyanobacteria [271]. Caldariella-quinone, which contains sulphur, has recently been detected in the thermophile and acidophile *Caldariella acidophilum* and *Solfolobus* [272].

Isoprenoid quinones are ideal taxonomic markers [102]. In recent years it has become clear that different bacteria produce various types of quinones, and that the number of isoprene units in the multiprenyl side-chain often differs among taxa. Isoprenoid quinones are of taxonomic importance in a number of Gram-positive eubacteria, such as *Bacillus*, *Listeria*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Thermoactinomyces*, *Actinomyces* and coryneforms, and also in many Gram-negative eubacteria, e.g., acetic acid bacteria, *Bacteroides*, *Campylobacter*, *Flavobacterium*, *Haemophilus*, *Legionella*, *Pasteurella*, *Pseudomonas* and the phototrophic bacteria *Thiobacillus* (for a review see ref. 271). It is also possible that the isoprenoid quinones may assist in the classification of archaebacteria. In addition to the distribution of various classes of respiratory quinones, and variations in the length of the multiprenyl side-chains of respiratory quinones, differences in the degree and position of hydrogenation of the multiprenyl side-chain (menaquinones) are of taxonomic importance, as seen in *Actinomadura*, *Streptomyces*, *Nocardia autrophica* and *N. brasiliensis* [102].

Isoprenoid quinones are rapidly photo-oxidized and susceptible to alkaline conditions. Extraction and purification should therefore be conducted rapidly, avoiding extreme pH values and strong light. Extraction is usually performed from dry cells with chloroform—methanol [273]. Quinones can also be extracted directly from wet cells with acetone [274]. Bacterial quinones can be separated by means of partition chromatography [102]. Reversed-phase partition paper chromatography, which previously was used extensively for separation of quinones, has now been superseded by reversed-phase TLC. Quantitative quinone profiles can be obtained by reversed-phase HPLC, or alternatively with silver ion-exchange chromatography [102]. The class or category to which an unknown isoprenoid quinone belongs can also be determined by ultraviolet spectroscopy. MS and NMR spectrometry are the most powerful methods for structural determinations of isoprenoid quinones, which also determine the exact site of saturation in the isoprenoid chain of partially hydrogenated menaquinones.

Carotenoids are produced by a variety of microorganisms and can be used as taxonomic markers [275]. Non-photosynthetic bacteria such as *Staphylococcus aureus* produce unique C_{30} carotenoids, e.g., 4-hydroxy-4,4′-diaponeurosporene. C_{40} carotenoids, including aliphatic, monocyclic, bicyclic, aromatic, phenolic, ketonic, hydroxylated and glycosylated derivatives, have a wide occurrence [276]. *Xanthomonas* strains may be differentiated from *Pseudomonas* strains through the presence or absence of brominated or non-brominated xanthomonadin pigments [277,278]. *Flexibacter*- and *Cytophaga*-like bacteria, *Sporocytophaga* and flavobacteria contain flexirubin-type pigments, which are related to carotenoids. The chemistry of carotenoids from phototrophic bacteria has been reviewed by Liaaen-Jensen [279], from fungi by Goodwin [280] and from algae by Liaaen-Jensen [281,282] and Goodwin [280].

Extraction of carotenoids has been described in Section 2.2. Carotenoids are non-volatile, relatively unstable substances. TLC and HPLC are usually

preferred when carotenoids are separated analytically. The computer-optimized normal-phase HPLC separation of *Corynebacterium poinsettiae* carotenoids demonstrated a mixture of new carotenoids for this organism [283].

4. BACTERIAL NUCLEIC ACIDS FOR CHEMOTAXONOMY

4.1. DNA reassociation and RNA hybridization

DNA reassociation or RNA hybridization experiments involve measurements of the pairing of two DNA fragments or the pairing of an RNA molecule with a DNA fragment [284]. Pairing occurs between the base pairs adenine and thymine, or uracil in RNA, and between guanine and cytosine. Reassociation or hybridization reactions give comparative measurements of the nucleotide sequence similarity. Differences here are the results of inversions, transitions, deletions and additions that have occurred in the past and may therefore reflect phylogenetic relationships. Nucleotide sequence similarities may range from 100% homology to no measurable similarity (0% homology). HPLC has become a very popular method for the analysis of nucleotides and nucleosides. DNA—DNA hybridization studies are primarily of importance in assessing the relationships within and between species [285]. DNA reassociation studies are suitable for establishing and evaluating taxonomies at the generic level in the case of overclassified taxa and the rank of species in most genera [4]. They are also valuable for detecting new centres of variation [286], and have provided a more unifying concept of a bacterial species. Homology levels for species inclusion have been suggested [287,288]. Owen and Pitcher [285] and Johnson [289] have described current methods for determining levels of DNA—DNA hybridization.

Several methods, e.g., the use of detergents, digestion with enzymes and physical procedures, have been used to disrupt bacterial cells for the extraction of nucleic acids. For the isolation of DNA, the Marmur method [290] and the hydroxyapatite method [284] are most often used. The hydroxyapatite method may replace the Marmur method when contamination with a polysaccharide is a problem. RNA can be isolated by the Kirby method [284], which is suitable for the isolation of bacterial ribosomal RNA essentially free from DNA and messenger RNA, and contains very little transfer RNA. Nucleic acid solutions are quantitated by measuring the absorbance at 260 nm, but colorimetric and fluorimetric assays can also be used. Nucleic acids can be compared by immobilization on a solid support. Reassociation, hybridization kinetics and DNA reassociation techniques have been described by Johnson [289].

4.2. DNA base composition

Direct analysis of the chromosomal DNA complement of the microbial cell has been a valuable approach to microbial classification [285]. DNA from most organisms contains the purine bases adenine (A) and guanine (G) and the pyrimidine bases thymine (T) and cytosine (C). The molar percentage of the G + C content of DNA from the prokaryotes ranges from about 25 to 75%,

while for most eukaryotes it is in the mid-forties [290]. The value is consistent for a given organism. The G + C content can be used as an excluding characteristic in bacterial taxonomy. This implies that if two organisms have DNA with different G + C contents, the organisms are different. Two organisms that have similar molar percentage G + C values are not necessarily closely related. The reason for this is that the molar percentage G + C values do not take into account the linear arrangement of the nucleotides in the DNA. Usually, however, organisms with a similar G + C content may be the same or similar. Conventional tests measure at best only 10% of the genetic capability of a bacterium [291]. Therefore, the study of DNA is particularly relevant for groups of bacteria where few tests are available for distinguishing between species. Current methods for determining DNA base compositions have been described by Owen and Pitcher [285]. Bacteria with DNA differing more than 5% in their G + C content should not be classified in the same species and those showing differences of more than 10% should not be assigned to the same genus [4].

Several methods have been used for DNA isolation. A rapid method for the isolation of small amounts of RNA-free DNA was recently described [284]. This procedure is best fitted for bacteria readily disrupted by a French press. Buoyant density centrifugation works best with high-molecular-weight DNA, and base or nucleotide chromatography and spectrophotometric analysis require DNA that is not contaminated with RNA. The thermal melting profile procedure is not particularly sensitive to RNA contamination. TLC and electrophoresis have also been used to separate DNA bases. A recent method for determining the G + C content of DNA has been to double-stain bacterial cells with the fluorescent dyes chromomycin A3 and Hoechst 33258, which bind to DNA rich in G + C and A + T, respectively. A dual-beam flow cytometer is then used to analyse the cells [292].

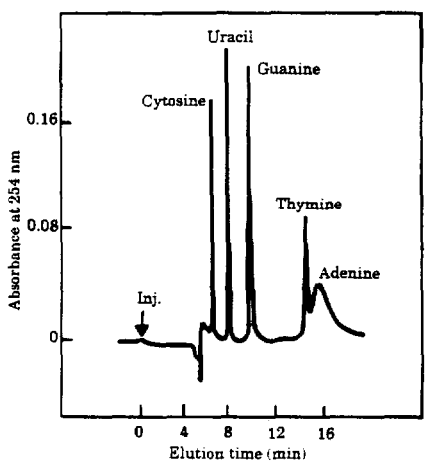

Fig. 6. Separation of pyrimidine and purine bases by high-performance reversed-phase chromatography. Sample: guanine, cytosine, adenine, uracil and thymine (Sigma), 0.5 nmol each in water with KOH added dropwise to dissolve fully. Mobile phase: 0.4 *M* $NH_4H_2PO_4$, pH 3.8, 5% (v/v) methanol. Flow-rate: 500 µl/min. Detection: 254 nm, 0.32 a.u.f.s. Injection volume: 20 µl. Temperature: 25°C. Pressure: 37 bar (3.7 MPa). (Courtesy LKB Produkter AB, Bromma, Sweden.)

Before analyses of DNA or RNA, their purity should be checked. RNA may be contaminated with proteins and polysaccharides. Methods for determining the purity of RNA have been described by Butterworth [293] and that of DNA by Marmur [290] and Maniatis et al. [294].

Purine and pyrimidine bases are of primary importance in biological systems, as components of nucleic acids. Reversed-phase chromatography is a powerful tool in nucleic acid research, where it can be used, for example, to determine the relationship between the DNA bases in different microorganisms and to determine the nucleic acid base composition of DNA and RNA species. The separation of pyrimidine and purine bases is shown in Fig. 6.

4.3. DNA restriction endonuclease fingerprinting

The organization of the microbial genotype may be assessed directly by means of restriction endonucleases [295]. This is a novel approach to the identification of microorganisms. Restriction endonucleases cleave DNA at specific sites and the resulting DNA fragments may be separated electrophoretically in gel to form specific restriction patterns. The potential of genome fingerprinting for differentiation between phenotypically closely related bacteria has remained largely unexplored until recently. The method has now proved useful for differentiation between species of *Mycoplasma pneumonia* [296], *Vibrio cholera* [297], *Neisseria meningitidis* [298—300] and *Neisseria gonorrheae* [301, 302].

The separation of DNA fragments after digestion by restriction endonucleases is of great interest for both DNA-sequencing studies and for recombinant DNA procedures. Until recently, the only technique capable of separating DNA fragments with sufficient resolution was electrophoresis in agarose or polyacrylamide gel. High-performance gel permeation chromatography using LKB Blue Columns (LKB, Ultro Pac TSK SW columns) is a very suitable technique for the separation of such fragments. The main advantage of HPLC over electrophoresis is the ability to obtain, in a few hours, and in good yield, large amounts of all the DNA restriction fragments for further study (Fig. 7). The application of HPLC to DNA analyses and interrelated techniques, advances in recent techniques and prospects for the future have been given by Wells [303]. The use of restriction endonuclease and sequence determination was illustrated for *Escherichia coli*. Schallinger et al. [304] described a sedimentation field flow fractionation method for the preparation of plasmid DNA on a preparative scale.

4.4. Ribosomal nucleic acid homology

Through ribosomal RNA—DNA reassociation studies, data for establishing affinities between bacteria at both the generic and suprageneric levels have been obtained [305]. Base sequences of rRNA cistrons are more conserved than most genes forming the bacterial genome. Insight into the phylogenetic relationships among prokaryotes can therefore be provided [306].

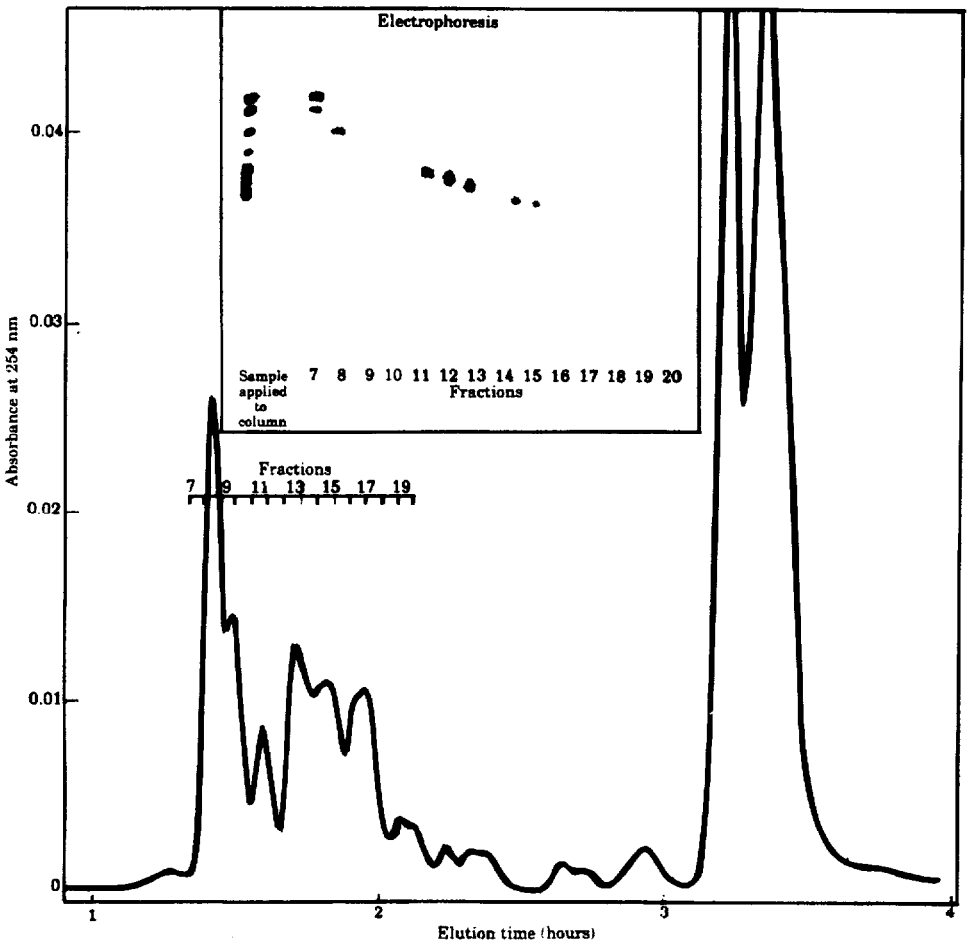

Fig. 7. Separation of DNA restriction fragments by high-performance gel permeation chromatography. Sample: Plasmid pBR322 cleaved by Hpa II, 10 μg fragments. Eluent: 50 m*M* ammonium acetate, pH 6.8. Flow-rate: 100 μl/min. Detection: 254 nm, 0.05 a.u.f.s. Injection volume: 100 μl. Temperature: 21°C. (Courtesy LKB Produkter AB, Bromma, Sweden.)

4.5. 16S ribosomal oligonucleotide cataloguing

Bacterial phylogenies have been constructed on the basis of similarities found between the nucleotide sequences of 16S rRNA [306]. 16S rRNA is a ubiquitous macromolecule, easily isolated, which exists as a major component of the small ribosomal subunit. It is ideal for studies in molecular systematics and is most suitable for phylogenetic studies as it can be used to determine close and very distant relationships (for a review, see ref. 307). After digestion of RNA with a nuclease of known specificity, the resulting oligonucleotides are separated in two dimensions and rendered visible by autoradiography (primary fingerprint). Thereafter, oligonucleotides in each spot of the fingerprint are sequenced in their entirety. Sequences of all nucleotides that comprise the original RNA are tabulated in a catalogue. Oligonucleotide catalogues from one organism, which give quantitative information obtained by actual determina-

tion of the 16S rRNA sequences, can be compared with that from another and similarly expressed by counting the number of unique or identical spots, or through association coefficients [308—310]. Procedures recommended for rRNA sequencing have been given by Fowler et al. [307]. The methods have been useful for the demonstration of the phylogeny of the genus *Actinomadura* [307]. A taxonomic study based solely on 16S rRNA has been demonstrated with methanogenic bacteria, which appeared as a unique group of organisms [311].

A general recipe for the isolation of 16S rRNA is not easy to give, as factors such as cell shape, age of cells, composition of the cell envelope, cell density and endonuclease activity tend to influence the isolation of pure rRNA. Stackebrandt et al. [310] have given a procedure that usually provides a satisfactory amount of pure 16S rRNA. Separation of rRNA from *Escherichia coli* is shown in Fig. 8.

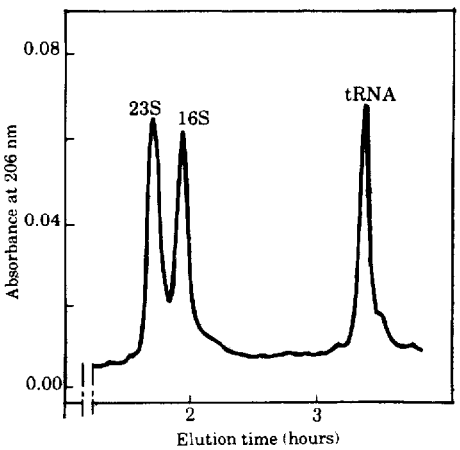

Fig. 8. Purification of ribonucleic acids by high-performance gel permeation chromatography. Sample: ribosomal RNA (*E. coli*) 16S and 23S, 10 μg each; tRNA (wheat germ), 9 μg. Eluent: 0.1 *M* Na_2HPO_4—NaH_2PO_4, 0.1 *M* NaCl, pH 6.8. Flow-rate: 100 μl/min. Detection: 206 nm, 0.1 a.u.f.s. Injection volume: 100 μl. Temperature: 22°C. (Courtesy LKB Produkter AB, Bromma, Sweden.)

Columns for high-performance gel permeation chromatography facilitate the separation of substances with a wide range of molecular weights. In this application, TSK-G 4000 SW grade can be used to analyse high-molecular-weight rRNA species (mol.wt. 1 000 000 and 550 000), which were clearly separated from each other and from the smaller tRNA in 3.5 h.

4.6. Genome size, plasmids and gene transfer

Bacteria can also be distinguished from their genome size, i.e., by the amount of chromosomal DNA they contain, expressed in daltons (incorrectly) or in the numbers of nucleotide pairs [312].

Although plasmids are widely distributed among bacteria, few attempts have been made to elucidate the distribution of extrachromosomal DNA for taxonomic purposes [4]. Plasmid profiles have been found useful in the identification of lactic streptococci [313] and in epidemiological studies of *Staphylococcus epidermidis* [314] and Gram-negative bacilli [315].

Domaradskiĭ [316] discussed the influence of plasmids on bacterial evolution. The presence of plasmids in bacteria may markedly change their biochemical properties. *Actinobacillus actinomycetemcomitans* strain FDC Y4, harbours a plasmid [317]. This may explain the complexity of the extractable lipids [123] and soluble protein pattern [248] of this organism in comparison with other strains of *A. actinomycetemcomitans*. Plasmid infection will also exclude phage induction. Exclusion of phage has been reported for Y4, contrary to other strains of *A. actinomycetemcomitans* [318].

Gene transfer, which can be obtained through transduction, transformation or conjugation, may be used to establish taxonomic data [319]. Generally, gene transfer and recombination can be achieved more frequently between members of closely related taxa than between members of groups more distantly related. On the other hand, a lack of ability to exchange genes may be related to factors other than failing genetic homology [320].

4.7. Phage host range studies

Phage host range studies may be used to obtain information on the similarities between bacterial strains. Thus a number of polyvalent phages have been used to detect lytic patterns at the generic level of bacteria [321].

5. SEROLOGY

Bacterial cell antigens used in serological studies include flagella, pili, cell walls, cytoplasmic membranes, capsules and slime layers. Serochemical specificity and locations of antigens in the bacterial cell have been discussed by Cummins [322]. *Enterobacteriaceae* are probably the organisms that have been most frequently subjected to serological studies. Thus, *Salmonella* contain more than 1000 serovars. However, these do not represent separate taxospecies, and the taxonomic value of such groups is therefore limited [255].

As there is a good correlation between the amino acid sequences of protein and the degree of serological similarity, antisera against purified enzymes have been used to study the homology of bacteria, e.g., the muconate-lactonizing enzymes of *Pseudomonadaceae*, the fructose diphosphate aldolases of the lactic acid bacteria and the catalases of staphylococci and *Micrococcaceae* [255]. Serological homology studies with proteins are very valuable for proteins with relatively high ($\geqslant 70\%$) sequence homologies. Although there are other limitations to the technique, serological studies provide a useful method for assessing structural homologies between proteins, and may therefore assist in the classification of bacteria and the elucidation of their phylogenetic relationships.

6. EXOGENOUS SUBSTANCES FOR CHEMOTAXONOMY

6.1. Biotransformation

Another attractive area for differentiation between clinical isolates was recently suggested [323,324]: by means of GC—MS, the biotransformation of nicotinic acid into 6-hydroxynicotinic acid was determined. Such a transforma-

tion has previously been described for species of *Clostridium* [325,326], *Bacillus* [327] and *Pseudomonas fluorescens* [328]. Maeda et al. [324] used the presence of hydroquinone in fermentation end-products as a criterion for the detection of *Escherichia coli*, which could be differentiated from *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa*. Hayward [28] described the rapid diagnosis of urinary tract infection by means of HSGC. The test depends on the production of ethanol from lactose or arabinose by *Escherichia coli* and some related species, and of methanethiol from methionine by *Proteus* spp., and is a modification of previously published methods [329,330]. The detection of trimethylamine produced from acetylcholine by *Proteus* spp. and some *Klebsiella aerogenes* has been described [27]. *K. aerogenes* was differentiated from *Proteus* spp. by ethanol production from arabinose. Ethyl acetate was produced from acetylcholine by *P. mirabilis*, *P. vulgaris*, *P. rettgeri*, *P. inconstans* A, *P. inconstans* B, *E. coli*, *K. aerogenes* and *Streptococcus faecalis*. Biotransformation or fermentation of exogenous substances such as glucose, galactose and xylose to acids or alcohols is routinely used as a biochemical test in bacterial classification and identification. In the above studies relatively unusual nutrient substances are biotransformed to metabolites that can be identified by chromatographic methods. Whereas fermentation tests require 24—48 h, biotransformation of the nutrient substance can be performed within 3—4 h. The fact that the assessment of end-products can be performed qualitatively with high precision makes this an attractive method in clinical microbiology.

7. SUMMARY

This review deals with the chemistry of marker substances used in microbial classification and identification, their isolation and purification and their biomedical application. A critical evaluation of current methods is also included. The information presented is partly based on personal experience, partly derived from more than 300 publications in this rapidly expanding field of science. Much has been done to improve the recognition of microorganisms by GC, but there are also a series of other techniques available that can assist in bacterial classification and identification. Some of these techniques have been made available to the clinical microbiologist through commercial systems, e.g., assessment of bacterial fatty acids. A fingerprint library has been developed by Hewlett-Packard for the analysis of fatty acids from approximately 6000 different bacteria. Other chemotaxonomic methods require great personal expertise and advanced equipment. Efforts should therefore be made to adapt and simplify such methods for application in the routine clinical laboratory. Chemical markers will probably have a great impact on future microbial taxonomy, particularly in cases where conventional methods fail to give satisfactory classifications. In order to make taxonomy more objective, there seems to be a need for screening of chemical markers in bacterial species and for compiling chemotaxonomic fingerprints in clinical manuals.

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REFERENCES

1 V.B.D. Skerman, V. McGowan and P.H.A. Sneath, Int. J. Syst. Bacteriol., 30 (1980) 225.
2 R.F. Taylor, Microbiol. Rev., 48 (1984) 181.
3 G.E. Fox, E. Stackebrandt, R.B. Hespell, J. Gibson, J. Maniloff, T.A. Dyer, R.S. Wolfe, W.E. Balch, R.S. Tanner, L.J. Magrum, L.B. Zablen, R. Blakemore, R. Gupta, L. Bonen, B.J. Lewis, D.A. Stahl, K.R. Luehrsen, K.N. Chen and C.R. Woese, Science, 209 (1980) 457.
4 M. Goodfellow and D.E. Minnikin, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 1–15.
5 I. Brondz and I. Olsen, J. Chromatogr., 374 (1986) 119.
6 I. Brondz and I. Olsen, J. Chromatogr., 380 (1986) 1.
7 M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985.
8 G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985.
9 N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams and Wilkins, Baltimore, MD, 1984.
10 M. Goodfellow and R.G. Board (Editors), Microbiological Classification and Identification, Academic Press, London, 1980.
11 D.B. Drucker, Microbiological Applications of Gas Chromatography, Cambridge University Press, London, 1981.
12 D.C. Edman and J.B. Brooks, J. Chromatogr., 274 (1983) 1.
13 I. Brondz, Dr. Thesis, University of Oslo, Oslo, 1985.
14 L.V. Holdeman, E.P. Cato and W.E.C. Moore (Editors), Anaerobe Laboratory Manual, 4th ed., Virginia Polytechnic Institute, Blacksburg, Va, 1977.
15 A.G. Deacon, B.I. Duerden and W.P. Holbrook, J. Med. Microbiol., 11 (1978) 81.
16 R. Hammann and H. Werner, in M. Goodfellow and R.G. Board (Editors), Microbiological Classification and Identification, Academic Press, London, 1980, pp. 257–271.
17 M.F. De La Cochetière-Collinet and L. Larsson, J. Chromatogr., 305 (1984) 178.
18 H.N. Shah, R.A. Nash, J.M. Hardie, D.A. Weetman, D.A. Geddes and T.W. MacFarlane, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 317–340.
19 M.B. Huysmans and J. Spicer, J. Chromatogr., 337 (1985) 223.
20 J.-L. Pons, A. Rimbault, J.C. Darbord and G. Leluan, J. Chromatogr., 337 (1985) 213.
21 L. Larsson, P.-A. Mårdh and G. Odham, Acta Pathol., Microbiol. Scand., 86B (1978) 207.
22 A. Tavakkol and D.B. Drucker, J. Chromatogr., 274 (1983) 37.
23 S.R. Dunn, M.L. Simenhoff and L.G. Wesson, Anal. Chem., 48 (1976) 41.
24 L. Thacker and J.B. Brooks, Infect. Immun., 9 (1974) 648.
25 J.B. Brooks and W.E.C. Moore, Can. J. Microbiol., 15 (1969) 1433.
26 J.B. Brooks, D.S. Kellogg, Jr., M.E. Shepherd and C.C. Alley, J. Clin. Microbiol., 11 (1980) 52.
27 T.J. Davies and N.J. Hayward, J. Chromatogr., 307 (1984) 11.
28 N.J. Hayward, J. Chromatogr., 274 (1983) 27.
29 F.A.J. Muskiet, G.A. van den Berg, A.W. Kingma, D.C. Freemann-Otterangers and M.R. Halie, Clin. Chem., 30 (1984) 687.

30 N. Seiler and B. Knödgen, J. Chromatogr., 339 (1985) 45.
31 P. Scherer and H. Kneifel, J. Bacteriol., 154 (1983) 1315.
32 L. Zoccolillo and M. Ronchetti, J. Chromatogr., 245 (1982) 321.
33 G.C. Cochrane, J. Chromatogr. Sci., 13 (1975) 440.
34 J. Pörschmann, T. Welsch, W. Engewald and G. Vigh, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 509.
35 I. Brondz, I. Olsen and T. Greibrokk, J. Chromatogr., 274 (1983) 299.
36 H.N.M. Ross, W.D. Grant and J.E. Harris, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 289—300.
37 M.M. Leahy and G.A. Reineccius, in P.S. Schreier (Editor), Analysis of Volatiles, Walter de Gruyter, Berlin, 1984, pp. 19—47.
38 S. Lowis, M.A. Eastwood and W.G. Brydon, J. Chromatogr., 278 (1983) 139.
39 R.F. Adams, R.L. Jones and P.L. Conway, J. Chromatogr., 336 (1984) 125.
40 S. Gal, M. Frommer-Filep, B.L. Toth-Martinez, F.J. Hernádi and L. Kiss, J. Chromatogr., 333 (1985) 239.
41 L. Kiss, B.L. Toth-Martinez, S. Gal and F.J. Hernádi, J. Chromatogr., 333 (1985) 244.
42 T. Sasagawa, L.H. Ericsson, D.C. Teller, K. Titani and K.A. Walsh, J. Chromatogr., 307 (1984) 29.
43 I. Brondz and I. Olsen, J. Chromatogr., 311 (1984) 31.
44 I. Brondz and I. Olsen, J. Chromatogr., 311 (1984) 347.
45 I. Brondz and I. Olsen, J. Chromatogr., 342 (1985) 13.
46 A.G. O'Donnell, D.E. Minnikin and M. Goodfellow, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 131—143.
47 E. Jantzen, K. Bryn and K. Bøvre, Acta Pathol. Microbiol. Scand., Sect. B, 82 (1974) 753.
48 L.A. Shute, R.C.W. Berkeley, J.R. Norris and C.S. Gutteridge, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 95—114.
49 G. Wieten, J. Haverkamp, H.L.C. Meuzelaar, H.B.V. Engel and L.G. Berwald, J. Gen. Microbiol., 122 (1981) 109.
50 G. Wieten, J. Haverkamp, L.G. Berwald, D.G. Groothuis and P. Draper, Ann. Microbiol. (Paris), 133B (1982) 15.
51 W. Windig, G.S. de Hoog and J. Haverkamp, J. Anal. Appl. Pyrol., 3 (1981) 213.
52 W. Windig and J. Haverkamp, Stud. Mycol., 22 (1982) 56.
53 W. Jennings, Gas Chromatography with Glass Capillary Columns, Academic Press, New York, 2nd ed., 1980, pp. 206—208.
54 G. Blomquist, E. Johansson, B. Söderström and S. Wold, J. Chromatogr., 173 (1979) 7.
55 G. Blomquist, E. Johansson, B. Söderström and S. Wold, J. Anal. Appl. Pyrol., 1 (1979) 53.
56 G. Blomquist, E. Johansson, B. Söderström and S. Wold, J. Chromatogr., 173 (1979) 19.
57 B. Söderström, S. Wold and G. Blomquist, J. Gen. Microbiol., 128 (1982) 1773.
58 G. Dahlén and I. Ericsson, J. Gen. Microbiol., 129 (1983) 557.
59 J. Chih-Au Hu, Adv. Chromatogr., 23 (1984) 149.
60 H. Mayer, R.N. Tharanathan and J. Weckesser, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 157—207.
61 I. Brondz and I. Olsen, J. Chromatogr., 310 (1984) 261.
62 E. Jantzen, K. Bryn and K. Bøvre, Acta Pathol. Microbiol. Scand., Sect. B, 84 (1976) 177.
63 M.P. Lechevalier, J. Lab. Clin. Med., 71 (1968) 934.
64 K.P. Schaal, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 359—381.
65 M.P. Lechevalier and H.A. Lechevalier, The Biology of the Actinomycetes, 11 (1976) 78.
66 A. Bacic, P.J. Harris, E.W. Hats and A.E. Clarke, J. Chromatogr., 315 (1984) 373.

67 B. Lindberg and J. Lønngren, Methods Enzymol., 50 (1978) 3.
68 J. Lønngren and S. Svensson, Adv. Carbohydr. Chem. Biochem., 29 (1974) 41.
69 N.K. Kochetkov, O.S. Chizhov and B.M. Zolotarev, Carbohydr. Res., 2 (1966) 89.
70 C.M. Park, P.E. Reid and D.A. Appelegarth, J. Chromatogr., 339 (1985) 182.
71 P.-E. Jansson, B. Lindberg, J. Lönngren, C. Ortega and W. Nimmich, Carbohydr. Res., 132 (1984) 297.
72 K.H. Schleifer and O. Kandler, Bacteriol. Rev., 36 (1972) 407.
73 F. Schumacher-Perdreau, H. Rotering and G. Pulverer, Zentralbl. Bakteriol. Parasitenkd., Infektionskr. Hyg., Abt. 1: Orig., Reihe A, 256 (1983) 25.
74 T.G. Tornabene, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 209—234.
75 T.A. Langworthy, G. Holzer, J.G. Zeikus and T.G. Tornabene, Syst. Appl. Microbiol., 4 (1983) 1.
76 H. Goldfine, Adv. Microbiol. Physiol., 8 (1972) 1.
77 D.E. Minnikin and A.G. O'Donnell, in M. Goodfellow, M. Mordarski and S.T. Williams (Editors), The Biology of the Actinomycetes, Academic Press, London, 1984, pp. 337—388.
78 T.J. Donohue, B.D. Cain and S. Kaplan, Biochemistry, 21 (1982) 2765.
79 E. Miyagawa, R. Azuma and T. Suto, J. Gen. Appl. Microbiol., 24 (1978) 341.
80 Y. Kawai, FEBS Lett., 153 (1983) 131.
81 Y. Kawai, K. Suzuki and T. Hagivara, Eur. J. Biochem., 147 (1985) 367.
82 T.A. Langworthy, W.R. Mayberry and P.F. Smith, Biochim. Biophys. Acta, 431 (1976) 550.
83 C.N. Kenyon, in R.K. Claton and W.R. Sistrom (Editors), The Photosynthetic Bacteria, Plenum, New York, 1978, pp. 281—313.
84 J.F. Imhoff, D.J. Kushner, S.C. Kushwaha and M. Kates, J. Bacteriol., 150 (1982) 1192.
85 W. Godchaux and E.R. Leadbetter, J. Bacteriol., 144 (1980) 592.
86 R.A. Pask-Hughes and N. Shaw, J. Bacteriol., 149 (1982) 54.
87 M. Oshima and T. Yamakawa, Biochemistry, 13 (1974) 1140.
88 T.A. Langworthy, W.R. Mayberry and P.F. Smith, J. Bacteriol., 119 (1974) 106.
89 T.A. Langworthy, T.G. Tornabene and G. Holzer, Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg., Abt. 1: Orig., Reihe C, 3 (1982) 228.
90 W.W. Christie, Lipid Analysis, Pergamon Press, Oxford, 2nd ed., 1982.
91 T.G. Tornabene, S.J. Morrison and W.E. Kloos, Lipids, 5 (1970) 929.
92 P. Bouvier, M. Rohmer, P. Benveniste and G. Qurisson, Biochem. J., 159 (1976) 267.
93 C.W. Bird, J.M. Lynch, S.J. Pirt, W.W. Reid, C.J.W. Brooks and B.S. Middleditch, Nature (London), 230 (1971) 473.
94 T.G. Tornabene, G. Holzer, A.S. Bittner and K. Grohmann, Can. J. Microbiol., 28 (1982) 1107.
95 M. DeRosa, A. Gambacorta and L. Minale, Phytochemistry, 12 (1973) 1117.
96 M.P. Lechevalier, CRC Crit. Rev. Microbiol., 5 (1977) 109.
97 O. Lüderitz, M.A. Freudenberg, C. Galanos, V. Lehmann, E.T. Rietschel and D.H. Shaw, Curr. Top. Membr. Transp., 17 (1982) 79.
98 D.E. Minnikin and M. Goodfellow, in M. Goodfellow and R.G. Board (Editors), Microbiological Classification and Identification, Academic Press, London, 1980, pp. 189—256.
99 G. Dobson, D.E. Minnikin, S.M. Minnikin, J.H. Parlett, M. Goodfellow, M. Ridell and M. Magnusson, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 237—265.
100 E. Jantzen and K. Bryn, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 145—171.
101 R.M. Kroppenstedt, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 173—199.
102 M.D. Collins, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 329—366.
103 E.G. Bligh and W.J. Dyer, Can. J. Biochem. Physiol., 37 (1959) 911.
104 M. Kates, G.A. Adams and S.M. Martin, Can. J. Biochem., 42 (1964) 461.

105 J. Folch, M. Lees and G.H. Sloan-Stanley, J. Biol. Chem., 226 (1957) 497.
106 P. Ways and D.J. Hanahan, J. Lipid Res., 5 (1964) 318.
107 J.N. Kaufer, Methods Enzymol., 14 (1969) 660.
108 M. Kates, in T.S. Work and E. Work (Editors), Techniques in Lipidology, Elsevier, Amsterdam, 1972, pp. 268—618.
109 I.J. Bousfield, G.L. Smith, T.R. Dando and G. Hobbs, J. Gen. Microbiol., 129 (1983) 375.
110 S. Liaaen-Jensen, in O. Isler (Editor), Carotenoids, Birkhäuser, Basle, 1971, pp. 61—188.
111 B.H. Davies, in T.W. Goodwin (Editor), Chemistry and Biochemistry of Plant Pigments, Vol. 2, Academic Press, New York, 1976, pp. 38—155.
112 E. De Ritter and A.E. Purcell, in L.C. Bauernfeind (Editor), Carotenoids as Colorants and Vitamin A Precursors, Academic Press, New York, 1981, pp. 815—924.
113 M.D. Collins, T. Pirouz, M. Goodfellow and D.E. Minnikin, J. Gen. Microbiol., 100 (1977) 221.
114 G.P. Hazlewood and R.M.C. Dawson, Biochem. J., 153 (1976) 49.
115 P. Zahler and V. Niggli, in E.D. Korn (Editor), Methods in Membrane Biology, Vol. 8, Plenum Press, New York, 1975, pp. 1—50.
116 F.C. Phillips and O.S. Privett, Lipids, 14 (1979) 590.
117 F.C. Phillips and O.S. Privett, Lipids, 14 (1979) 949.
118 M.A. Wells and J.C. Dittmer, Biochemistry, 2 (1963) 1259.
119 R.E. Wuthier, J. Lipid Res., 7 (1966) 558.
120 H.-W. Wollenweber, S. Schlecht, O. Lüderitz and E.T. Rietschel, Eur. J. Biochem., 130 (1983) 167.
121 A.T. James and A.J.P. Martin, Biochem. J., 50 (1952) 679.
122 K.I. Sakodynsky, G.A. Smolyaninov, V.Yu. Zelvensky and N.A. Glotova, J. Chromatogr., 172 (1979) 93.
123 I. Brondz and I. Olsen, J. Chromatogr., 278 (1983) 13.
124 J.M. Levy, L.E. Wolfram and J.A. Yancey, J. Chromatogr., 279 (1983) 133.
125 L. Julen, S.P. Enquist, Chromatographia, 17 (1983) 549.
126 I. Molnar-Perl and M. Szakácz-Purter, Chromatographia, 17 (1983) 328.
127 I. Molnar-Perl and M. Szakácz-Purter, Chromatographia, 17 (1983) 493.
128 K. Kimura, M. Sawada and T. Shono, J. Chromatogr., 240 (1983) 361.
129 D. Furestone and W. Horwitz, J. Assoc. Off. Anal. Chem., 62 (1979).
130 S. Hase, T. Hofstad and E.T. Rietschel, J. Bacteriol., 129 (1977) 9.
131 C.W. Moss, in G.L. Gilardi (Editor), Glucose Non-Fermenting Gram-Negative Bacteria in Clinical Microbiology, CRC Press, West Palm Beach, FL, 1978, pp. 171—201.
132 R.P. Hansen and J.E. Smith, Lipids, 1 (1966) 316.
133 C.W. Moss, M.A. Lambert and W.H. Mervin, Appl. Microbiol., 28 (1974) 80.
134 H. Schlenk and J.L. Gellerman, Anal. Chem., 32 (1960) 1412.
135 S. Bauer, M. Neupert and G. Spiteller, J. Chromatogr., 309 (1984) 243.
136 R. Wood and F. Snyder, Lipids, 1 (1966) 62.
137 R. Watts and R. Dils, Chem. Phys. Lipids, 3 (1969) 168.
138 J.J. Myher, in D.J. Hanahan and A. Kuksis (Editors), Handbook of Lipid Research, Vol. 1, Plenum Press, New York, pp. 123—196.
139 E.J. Corey, Jr. and A. Venkateswarlu, J. Am. Chem. Soc., 94 (1972) 6190.
140 I. Brondz and I. Olsen, J. Chromatogr., 308 (1984) 282.
141 L.J. Nutter and O.S. Privett, Lipids, 1 (1966) 234.
142 O. Renkonen, Biochim. Biophys. Acta, 125 (1966) 288.
143 D.P. Schwartz, Anal. Biochem., 71 (1976) 24.
144 O. Renkonen, J. Am. Oil Chem. Soc., 42 (1965) 298.
145 O. Renkonen, Lipids, 1 (1966) 160.
146 C.F. Poole and A. Zlatkis, J. Chromatogr. Sci., 17 (1979) 115.
147 C.F. Poole, in K. Blau and G.S. King (Editors), Handbook of Derivatives for Chromatography, Heyden, London, 1978, pp. 152—200.
148 W.H. Tallent and R. Kleiman, J. Lipid Res., 9 (1968) 146.
149 J. Drozd, J. Chromatogr., 113 (1975) 303.
150 R. Wood, Lipids, 2 (1967) 199.

151 G.M. Anthony, C.J.W. Brooks, I. MacLean and I. Sangster, J. Chromatogr. Sci., 7 (1969) 623.
152 C.J.W. Brooks and I. MacLean, J. Chromatogr. Sci., 9 (1971) 18.
153 R. Ryhage and E. Stenhagen, Ark. Kemi, 13 (1959) 523.
154 F.W. McLafferty, Anal. Chem., 31 (1959) 82.
155 B. Hallgren, R. Ryhage and E. Stenhagen, Acta Chem. Scand., 13 (1959) 845.
156 J.M.B. Apon and N. Nicolaides, J. Chromatogr. Sci., 13 (1975) 467.
157 J.A. McCloskey and J.H. Law, Lipids, 2 (1967) 225.
158 B.A. Andersson and R.T. Holman, Lipids, 10 (1975) 716.
159 J.A. McCloskey and M.J. McClelland, J. Am. Chem. Soc., 87 (1965) 5090.
160 C.J. Argoudelis and E.G. Perkins, Lipids, 3 (1968) 379.
161 P. Capella and C.M. Zorzut, Anal. Chem., 40 (1968) 1458.
162 V. Dommes, F. Wirtz-Peitz and W.H. Kunau, J. Chromatogr. Sci., 14 (1976) 360.
163 G. Janssen and G. Parmentier, Biomed. Mass Spectrom., 5 (1978) 439.
164 B. Schmitz and H. Egge, Chem. Phys. Lipids, 25 (1979) 287.
165 D.E. Minnikin, Lipids, 7 (1972) 398.
166 D.E. Minnikin, P. Abley, F.J. McQuillin, K. Kusamran, K. Maskens and N. Polgar, Lipids, 9 (1974) 135.
167 R.D. Platter, G.F. Spencer and R. Kleiman, Lipids, 11 (1976) 222.
168 A.J. Valicenti, W.H. Heimermann and R.T. Holman, J. Org. Chem., 44 (1979) 1068.
169 T.A. Eisele, L.M. Libbey, N.E. Pawloski, J.E. Nixon and R.O. Sinnhuber, Chem. Phys. Lipids, 12 (1974) 316.
170 N.K. Hooper and J.H. Law, J. Lipid Res., 9 (1968) 270.
171 P.K. Raju and R. Reiser, Lipids, 2 (1967) 197.
172 W.W. Christie, D. Rebello and R.T. Holman, Lipids, 4 (1969) 229.
173 V.K.S. Shukla, E.M. Abdel-Moety, E. Larsen and H. Egsgaard, Chem. Phys. Lipids, 23 (1979) 285.
174 J.C.M. Schogt and P. Haverkamp-Begemann, J. Lipid Res., 6 (1965) 466.
175 L.J. Morris, M.O. Marshall and W. Kelly, Tetrahedron Lett., (1966) 4249.
176 C.W. Moss and S.B. Dees, J. Chromatogr., 112 (1975) 595.
177 R.F. Adams, R.L. Jones and P.L. Conway, J. Chromatogr., 336 (1984) 125.
178 R.T. Marsili, J. Chromatogr. Sci., 19 (1981) 451.
179 G.G. Ehrlich, D.F. Goerlitz, J.H. Bourell, G.V. Eisen and E.M. Godsy, Appl. Environ. Microbiol., 42 (1981) 878.
180 G.O. Guerrant, M.A. Lambert and C.W. Moss, J. Clin. Microbiol., 16 (1982) 355.
181 R. Wood and T. Lee, J. Chromatogr., 254 (1983) 237.
182 M.J. Cooper and M.W. Anders, J. Chromatogr. Sci., 13 (1975) 407.
183 A.W. Reed, H.C. Deeth and D.E. Clegg, J. Assoc. Off. Anal. Chem., 67 (1984) 718.
184 A.G. Netting and A.M. Duffield, J. Chromatogr., 336 (1984) 115.
185 S.T. Ingalls, P.E. Minkler, C.L. Hoppel and J.E. Nordlander, J. Chromatogr., 299 (1984) 365.
186 N. Ichinose, K. Nakamura, C. Shimizu, H. Kurokura and K. Okamoto, J. Chromatogr., 295 (1984) 463.
187 R. Farinotti, P. Siard, J. Bourson, S. Kirkiacharian, B. Valeur and G. Mahuzier, J. Chromatogr., 269 (1984) 81.
188 H. Lingeman, A. Hulshoff, W.J.M. Underberg and F.B.J.M. Offermann, J. Chromatogr., 290 (1984) 215.
189 M. Ikeda, K. Shimada, T. Sakaguchi and U. Matsumoto, J. Chromatogr., 305 (1984) 261.
190 H. Miwa, C. Hiyama and M. Yamamoto, J. Chromatogr., 321 (1985) 165.
191 M.S.F. Lie Ken Jie, Adv. Chromatogr., 18 (1980) 1.
192 L.J. Morris, J. Lipid Res., 7 (1966) 717.
193 F.D. Gunstone, I.A. Ismail and M.S.F. Lie Ken Jie, Chem. Phys. Lipids, 1 (1967) 376.
194 S.A. Kibardin and K.A. Makarov, in M.N. Patushenko (Editor), Tonkosloinaya Khromatografia v Organicheskoi Khimii, Moscow, 1978, pp. 48—58.
195 V. Pollack, Adv. Chromatogr., 17 (1979) 1.
196 J. Weckesser, H. Mayer, G. Drews and I. Fromme, J. Bacteriol., 123 (1975) 449.

197 J. Weckesser, H. Mayer, G. Drews and I. Fromme, J. Bacteriol., 123 (1975) 456.
198 P.H. Mäkelä and B.A.D. Stocker, in E.T. Rietschel (Editor), Handbook of Endotoxin, Vol. 1, Elsevier, Amsterdam, 1984, pp. 59—137.
199 G. Schmidt and O. Lüderitz, Zentralbl. Bakteriol. Parasitenkd., Infektionskr. Hyg., Abt. 1: Orig., Reihe A, 210 (1969) 381.
200 G. Schmidt, S. Schlecht, O. Lüderitz and O. Westphal, Zentralbl. Bakteriol. Parasitenkd., Infektionskr. Hyg., Abt. 1: Orig., Reihe A, 210 (1969) 483.
201 H. Mayer and G. Schmidt, Zentralbl. Bakteriol. Parasitenkd., Infektionskr. Hyg., Abt. 1: Orig., Reihe A, 224 (1973) 345.
202 M.N. Ahamed, J. Radziejewska-Lebrecht, C. Widemann and H. Mayer, Zentralbl. Bakteriol. Parasitenkd., Infektionskr. Hyg., Abt. 1: Orig., Reihe A, 247 (1980) 468.
203 K. Kotelko, W. Gromska, M. Papierz, Z. Sidorczyk, D. Krajewska and K. Szer, J. Hyg. Epidemiol. Microbiol. Immunol., 21 (1977) 271.
204 S.V. Tomshich, R.P. Gorshkova and Y.S. Ovodov, in Abstracts of Second European Symposium on Carbohydrates and Glycoconjugates, Budapest, 1983, p. C-34.
205 E.T. Rietschel and O. Lüderitz, Forum Mikrobiol., 1 (1980) 12.
206 G. Drews, J. Weckesser and M. Mayer, in R.K. Clayton and W.R. Sistrom (Editors), The Photosynthetic Bacteria, Plenum, New York, 1978, p. 61.
207 E.T. Rietschel, H.-W. Wollenweber, H. Brade, U. Zähringer, B. Lindner, U. Seydel, H. Bradaczek, G. Barnickel, H. Labischinski and P. Giesbrecht, in E.T. Rietschel (Editor), Handbook of Endotoxin, Vol. 1, Elsevier, Amsterdam, 1984, pp. 187—220.
208 U. Seydel, B. Lindner, H.-W. Wollenweber and E.T. Rietschel, Biomed. Mass Spectrom., 11 (1984) 132.
209 H. Mayer and J. Weckesser, in E.T. Rietschel (Editor), Handbook of Endotoxin, Vol. 1, Elsevier, Amsterdam, 1984, pp. 221—247.
210 H. Mayer, in E. Haber (Editor), Festschrift für Herman M. Kalckar, Plenum, New York, 1984.
211 E. Seewaldt, K.H. Schleifer, E. Bock and E. Stackebrandt, Acta Microbiol., 131 (1982) 287.
212 E.T. Rietschel, Z. Sidorczyk, U. Zähringer, H.-W. Wollenweber and O. Lüderitz, ACS Symp. Ser., No. 231 (1983) 195.
213 H.-W. Wollenweber, U. Seydel, B. Lindner, O. Lüderitz and E.T. Rietschel, Eur. J. Biochem., 145 (1984) 265.
214 S.G. Wilkinson and P.F. Caudwell, J. Gen. Microbiol., 118 (1980) 329.
215 O. Westphal and K. Jann, Methods Carbohydr. Chem., 5 (1965) 83.
216 C. Galanos, O. Lüderitz and O. Westphal, Eur. J. Biochem., 9 (1969) 245.
217 G.A. Adams, Can. J. Chem., 45 (1967) 422.
218 R.P. Darveau and R.E.W. Hancock, J. Bacteriol., 115 (1983) 831.
219 W.T.J. Morgan, Methods Carbohydr. Chem., 5 (1965) 80.
220 A. Boivin and L. Mesrobeanu, C.R. Seances Soc. Biol. Ses. Fil., 112 (1933) 76.
221 O. Kandler and K.H. Schleifer, Prog. Bot., 42 (1980) 234.
222 K.H. Schleifer, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 123—156.
223 H. Hayashi, Y. Araki and E. Ito, J. Bacteriol., 113 (1973) 592.
224 E. Schmelzer, J. Weckesser, R. Warth and M. Mayer, J. Bacteriol., 149 (1982) 151.
225 D. Mirelman and N. Sharon, J. Biol. Chem., 242 (1967) 3414.
226 E. Lederer, A. Adam, R. Ciorbaru, J.-F. Petit and J. Wietzerbin, Mol. Cell. Biochem., 7 (1975) 87.
227 K. Uchida and K. Aida, J. Gen. Appl. Microbiol., 23 (1977) 249.
228 M. Goodfellow and T. Cross, in M. Goodfellow, M. Mordarski and S.T. Williams (Editors), The Biology of the Actinomycetes, Academic Press, London, 1984, pp. 7—164.
229 K.H. Schleifer and P.H. Seidl, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, New York, 1985, pp. 201—219.

230 K. Uchida and K. Aida, J. Gen. Appl. Microbiol., 25 (1979) 169.
231 D.E. Minnikin, P.V. Patel, L. Alshamaony and M. Goodfellow, Int. J. Syst. Bacteriol., 27 (1977) 104.
232 G.L. Card, J. Bacteriol., 114 (1973) 1125.
233 M.H. Conover, J.S. Thompson and G.D. Shockman, Biochem. Biophys. Res. Commun., 23 (1966) 713.
234 K.H. Schleifer, Z. Immunitätforsch., 149 (1975) 104.
235 T.J. Dougherty, J. Bacteriol., 163 (1985) 69.
236 S.A. Bitko, E.P. Savel'ev and G.I. Petrov, Prikl. Biokhim. Mikrobiol., 20 (1984) 285.
237 B. Glauner and U. Schwarz, in R. Hakenbeck, J.-V. Hölje and H. Zabischinski (Editors), The Target of Penicillin, Walter de Gruyter, Berlin, 1983, pp. 29—34.
238 W. Weidel, H. Frank and H.H. Martin, J. Gen. Microbiol., 22 (1960) 158.
239 V. Braun and U. Sieglin, Eur. J. Biochem., 13 (1970) 336.
240 S.D. Goodwin and J.C. Shedlarski, Arch. Biochem. Biophys., 170 (1975) 23.
241 U.J. Jürgens, G. Drews and J. Weckesser, J. Bacteriol., 154 (1983) 471.
242 P.J.H. Jackman, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 115—129.
243 K. Kersters and J. De Ley, in M. Goodfellow and R.G. Board (Editors), Microbiological Classification and Identification, Academic Press, London, 1980, pp. 273—297.
244 P.J.H. Jackman, in C.S. Gutteridge (Editor), New Methods for the Detection and Characterisation of Micro-organisms, Wiley, Chichester, 1984.
245 J. Swings, K. Kersters and J. De Ley, J. Gen. Microbiol., 93 (1976) 266.
246 R.A.D. Williams and H.N. Shah, in M. Goodfellow and R.G. Board (Editors), Microbiological Classification and Identification, Academic Press, London, 1980, pp. 299—318.
247 P.H. O'Farell, J. Biol. Chem., 250 (1975) 4007.
248 E. Jellum, V. Tingelstad and I. Olsen, Int. J. Syst. Bacteriol., 34 (1984) 478.
249 G.P. Roberts, W.T. Leps, L.E. Silver and W.J. Brill, Appl. Environ. Microbiol., 39 (1980) 414.
250 C. Mouches, J.C. Vignault, J.G. Tully, R.F. Whitcomb and J.M. Bové, Curr. Microbiol., 2 (1979) 69.
251 F.C. Neidelhardt, V. Vaughn, T.A. Phillips and P.L. Bloch, Microbiol. Rev., 47 (1983) 231.
252 A. Böck, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 109—122.
253 M.A. Carver and C.W. Jones, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 383—399.
254 C.W. Jones and R.K. Poole, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 285—328.
255 D. Jones and N.R. Krieg, in N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams and Wilkins, Baltimore, MD, 1984, pp. 15—18.
256 R.P. Ambler, in G.D. Tasman (Editor), Handbook of Biochemistry and Molecular Biology, Vol. 3, CRC Press, Cleveland, OH, 1976, pp. 292—307.
257 R.P. Ambler, T.E. Meyer and M.D. Kamen, Nature (London), 278 (1979) 661.
258 R.P. Ambler, in N.O. Kaplan and A. Robinson (Editors), From Cyclotrons to Cytochromes, Academic Press, New York, 1980, pp. 263—280.
259 S. Fleischer and L. Packer (Editors), Methods in Enzymology, Vol. 54, Academic Press, New York, 1978.
260 I. Brondz and I. Olsen, 2nd European Congress of Clinical Microbiology, Brighton, 1985, Abstr. 21/9.
261 S. Bascomb, in M. Goodfellow and R.G. Board (Editors), Microbiological Classification and Identification, Academic Press, London, 1980, pp. 359—373.
262 I. Brondz and I. Olsen, 13th International Congress in Biochemistry, Amsterdam, 1985, Abstract TU 292, p. 304.
263 R.E. Doolittle, Science, 214 (1981) 149.
264 R.P. Ambler, in M. Goodfellow, D. Jones and F.G. Priest (Editors), Current Topics in Computer-Assisted Bacterial Systematics, Academic Press, New York, 1985.

265 R.-L. Cunico, R. Simpson, L. Correia and C.T. Wehr, J. Chromatogr., 336 (1984) 105.
266 Z. Deyl, J. Chromatogr., 127 (1976) 91.
267 B.A. Bidlingmeyer, S.A. Cohen and T.L. Tarvin, J. Chromatogr., 336 (1984) 93.
268 P. Giliberti and A. Niederwieser, J. Chromatogr., 66 (1972) 261.
269 K. Otsuka, S. Terabe and T. Ando, J. Chromatogr., 332 (1985) 219.
270 S. Keller, A.K. Ghosh, A.K. Ghosh, M. Turino and I. Mandl, J. Chromatogr., 305 (1984) 461.
271 M.D. Collins and D. Jones, Microbiol. Rev., 45 (1981) 316.
272 M.D. Collins and T.A. Longworthy, Syst. Appl. Microbiol., 4 (1983) 295.
273 M.D. Collins, T. Pirouz, M. Goodfellow and D.E. Minnikin, J. Gen. Microbiol., 100 (1977) 221.
274 R.M. Kroppenstedt, GIT Labor-Med., 4 (1982) 266.
275 S. Liaaen-Jensen, Pure Appl. Chem., 51 (1979) 661.
276 S. Liaaen-Jensen and A.G. Andrewes, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 235—255.
277 M.P. Starr, C.L. Jenkins, L.B. Bussey and A.G. Andrewes, Arch. Microbiol., 113 (1977) 1.
278 C.L. Jenkins and M.P. Starr, Curr. Microbiol., 7 (1982) 323.
279 S. Liaaen-Jensen, in R.K. Clayton and W.R. Sistrom (Editors), Photosynthetic Bacteria, Plenum, New York, 1978, pp. 233—248.
280 T.W. Goodwin, The Biochemistry of the Carotenoids, Vol. 1, Plants, 2nd ed., Chapman and Hall, London, 1980.
281 S. Liaaen-Jensen, in D.J. Faulkner and W.H. Fenical (Editors), Marine Natural Products Chemistry, Plenum, New York, 1977, pp. 225—237.
282 S. Liaaen-Jensen, in P. Scheuer (Editor), Marine Natural Products. Chemical and Biological Perspectives, Vol. 2, Academic Press, New York, 1978, pp. 2—73.
283 A.S. Kester and R.E. Thompson, J. Chromatogr., 310 (1984) 372.
284 J.L. Johnson, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 33—74.
285 R.J. Owen and D. Pitcher, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 67—93.
286 V.E. Dent and R.A.D. Williams, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 341—357.
287 J.L. Johnson, Int. J. Syst. Bacteriol., 23 (1973) 308.
288 J.L. Johnson, in N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams and Wilkins, Baltimore, MD, 1984, pp. 8—11.
289 J.L. Johnson, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 1—31.
290 J. Marmur, J. Mol. Biol., 3 (1961) 208.
291 S. Harvey and M.J. Pickett, Int. J. Syst. Bacteriol., 30 (1980) 86.
292 M.A. Van Dilla, R.G. Langlois, D. Pinkel, D. Yajko and W.K. Hadley, Science, 220 (1983) 620.
293 P.H.W. Butterworth, in G.D. Birnie (Editor), Subnuclear Components, Preparation and Fractionation, Butterworths, London, 1976, pp. 295—323.
294 T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Publications, New York, 1982.
295 E.M. Southern, Biochem. Soc. Symp., 44 (1979) 37.
296 D.K.F. Chandler, S. Razin, E.B. Stephens, R. Harasawa and M.F. Barile, Infect. Immun., 38 (1982) 604.
297 J.B. Kaper, H.B. Bradford, N.C. Roberts and S. Falkow, J. Clin. Microbiol., 16 (1982) 129.
298 B. Bjorvatn, V. Lund, B.-E. Kristiansen, L. Korsnes, O. Spanne and B. Lindquist, J. Clin. Microbiol., 19 (1984) 763.
299 B.-E. Kristiansen, B. Sørensen, T. Simonsen, O. Spanne, V. Lund and B. Bjorvatn, J. Infect. Dis., 150 (1984) 389.

300 B.-E. Kristiansen, B. Sørensen and B. Bjorvatn, Natl. Inst. Publ. Health Ann., 7 (1984) 21.
301 E.S. Falk, B. Bjorvatn, D. Danielsson, B.-E. Kristiansen, K. Melby and B. Sørensen, Acta Pathol. Microbiol. Immunol. Scand., Sect. B, 92 (1984) 271.
302 B.-E. Kristiansen, B. Sørensen, O. Spanne and B. Bjorvatn, Scand. J. Infect. Dis., 17 (1985) 19.
303 R.D. Wells, J. Chromatogr., 336 (1984) 3.
304 L.E. Schallinger, J.E. Gray, L.W. Wagner, S. Knowlton and J.J. Kirkland, J. Chromatogr., 342 (1985) 67.
305 M. Mordarski, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 41—66.
306 C.R. Woese, M. Sogin, D. Stahl, B.J. Lewis and L. Bonen, J. Mol. Evol., 7 (1976) 197.
307 V.J. Fowler, W. Ludwig and E. Stackebrandt, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, Academic Press, London, 1985, pp. 17—40.
308 C.R. Woese, M.L. Sogin and L.A. Sutton, J. Mol. Evol., 3 (1974) 293.
309 L. Zablen, L. Bonen, R. Meyer and C.R. Woese, J. Mol. Evol., 4 (1975) 347.
310 E. Stackebrandt, W. Ludvig and G.E. Fox, in G. Gottschalk (Editor), Methods in Microbiology, Vol. 18, Academic Press, London, 1985, pp. 75—107.
311 C.R. Woese and G.F. Fox, Proc. Natl. Acad. Sci. U.S.A., 74 (1977) 5088.
312 R.J. Owen and L.R. Hill, in F.A. Skinner and D.W. Lovelock (Editors), Identification Methods for Microbiologists, Academic Press, London, 2nd ed., 1979, pp. 277—296.
313 F.L. Davies, H.M. Underwood and M.J. Gasson, J. Appl. Bacteriol., 51 (1981) 325.
314 J.T. Parisi and D.W. Hecht, J. Infect. Dis., 141 (1980) 637.
315 D.R. Schaberg, L.S. Tompkins and S. Falkow, J. Clin. Microbiol., 13 (1981) 1105.
316 I.V. Domaradskiĭ, J. Evol. Biochem. Physiol., 19 (1983) 3.
317 B.F. Hammond, K. Peindl and S.S. Socransky, Int. Assoc. Dent. Res. Abstr., 1975, Abstract L217, p. L55.
318 R.H. Stevens, B.F. Hammond and C.H. Lai, Infect. Immun., 35 (1982) 343.
319 K. Bøvre, in M. Goodfellow and R.G. Board (Editors), Microbiological Classification and Identification, Academic Press, London, 1980, pp. 55—72.
320 K. Sakaguichi, in K. Sakaguichi and M. Okanishi (Editors), Molecular Breeding and Genetics of Applied Microorganisms, Academic Press, London, 1980, pp. 1—7.
321 H. Prauser, in L. Bojalil and L. Ortiz-Ortiz (Editors), Actinomycete Biology, Academic Press, New York, 1984.
322 C.S. Cummins, in G.C. Ainsworth and P.H.A. Sneath (Editors), Microbial Classification, 12th Symposium of the Society for General Microbiology, Cambridge University Press, 1962.
323 S. Shiraishi, N. Sakamoto, K. Maeda, T. Ohki, M. Hosoi, K. Ohta and N. Yamanaka, J. Chromatogr., 338 (1985) 51.
324 K. Maeda, S. Shiraishi, N. Sakamoto, T. Ohki, M. Hosoi, K. Ohta and N. Yamanaka, J. Chromatogr., 345 (1985) 11.
325 I.J. Havary, J. Biol. Chem., 227 (1957) 815.
326 I.J. Havary, J. Biol. Chem., 227 (1957) 823.
327 J.C. Ensinn and S.C. Rittenberg, J. Biol. Chem., 239 (1984) 2285.
328 D.E. Hughes, Biochem. J., 60 (1955) 303.
329 N.J. Hayward and T.H. Icavons, J. Clin. Microbiol., 6 (1977) 202.
330 P.J. Coloe, J. Clin. Pathol., 31 (1978) 365.