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Polyphasic Taxonomy, a Consensus Approach to Bacterial Systematics

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INTRODUCTION

For a long time, bacterial taxonomy was considered one of the dullest fields in microbiology, not immediately the preferred discipline of young or ambitious scientists. Recent developments have changed this attitude, mainly because of the spectacular developments witnessed in the last 10 years in the field of sequencing of rRNA and genes coding for rRNA (rDNA) and their contribution to bacterial phylogeny and in molecular fingerprinting techniques. These techniques revolutionized our insights in the phylogeny and taxonomy of all living organisms. Taxonomy of bacteria finally also could be assigned a place in phylogeny.

Another development of bacterial taxonomy, polyphasic taxonomy, arose 25 years ago and is aiming at the integration of different kinds of data and information (phenotypic, genotypic, and phylogenetic) on microorganisms and essentially indicates a consensus type of taxonomy. The term "polyphasic taxonomy" was coined by Colwell (45) and is used for the delineation of taxa at all levels (219). Also, the terms "polyphasic classification" and "polyphasic identification" can be validly used in this context. The recent developments of polyphasic taxonomy and phylogeny clearly constitute milestones in modern bacterial taxonomy. There will never be a definitive classification of bacteria. But let us be clear: this is not meant as a "fin de siècle" pessimistic statement of postmodernists among the choir of jubilating optimists! It is only the illustration of a rule, valid in all experimental sciences, stating that scientific progress is linked to and made possible through technological progress.

In the three parts of the present contribution on polyphasic taxonomy, we will successively (i) discuss the types of information used, (ii) illustrate the practice of polyphasic taxonomy in a selected number of cases, and (iii) discuss its problems and future developments. For overviews of modern taxonomic theory and practice, we refer to recent handbooks, e.g., by Priest and Austin (257), Goodfellow and O'Donnell (112), Towner and Cockayne (304), and Logan (187), and general works, e.g., *The Prokaryotes* (8) and *Bergey's Manual of Systematic Bacteriology* (172).

POLYPHASIC TAXONOMY

Taxonomy is generally taken as a synonym of systematics or biosystematics and is traditionally divided into three parts: (i) classification, i.e., the orderly arrangement of organisms into taxonomic groups on the basis of similarity; (ii) nomenclature, i.e., the labelling of the units defined in (i); and (iii) identification of unknown organisms, i.e., the process of determining whether an organism belongs to one of the units defined in (i) and labeled in (ii) (51, 292). Two additional parts are needed to completely define modern biosystematics: phylogeny and population genetics. In the last decade, it became generally accepted that bacterial classification should reflect as closely as possible the natural relationships between bacteria, which are the phylogenetic relationships as encoded in 16S or 23S rRNA sequence data (363).

The species is the basic unit of bacterial taxonomy (343) and is defined as a group of strains, including the type strain, sharing 70% or greater DNA-DNA relatedness with 5°C or

less ΔT_m (T_m is the melting temperature of the hybrid as determined by stepwise denaturation; ΔT_m is the difference in T_m in degrees Celsius between the homologous and heterologous hybrids formed under standard conditions [343]). Phenotypic and chemotaxonomic features should agree with this definition. The designated type strain of a species serves as the name bearer of the species and as the reference specimen (292). Before this definition was generally accepted, Staley and Krieg (292) defended a much more vague species concept that consisted of the type strain and all other strains considered sufficiently similar to this type to warrant their inclusion within a single species. The bacterial species definition given above is founded upon whole genomic DNA-DNA hybridization values (343). Practical problems exist, however, because different methods are used to determine the level of DNA-DNA hybridization. These methods do not always give the same (quantitative) results, and the value of 70% DNA relatedness seems only to be indicative rather than absolute (see below). Although not available, an alternative phylogenetic species concept could delineate a species in a phylogenetic framework as determined by percent 16S rRNA similarities.

Bacterial taxonomists, asked about their ideas about the bacterial species, are caught between Scylla and Charybdis: either they stick to a coherent species definition without it necessarily being a biological reality, or they visualize bacterial species as condensed nodes in a cloudy and confluent taxonomic space. The latter view implies that classification is a frame for the condensed nodes where some isolated internodal strains must also get a (provisional) place and name. Loosening the 70% rule often allows a compromise between the two views (see below).

Within the present manuscript, all the attention will be focused on the taxonomic ranks of species, genus, and family. The species is certainly the most important and at the same time the central element of bacterial taxonomy, but the hierarchical structure of taxonomy requires us to consider at least the higher taxa of genus and family. Much more than the species, they are difficult to define and represent agglomerates of nodal species and internodal strains and agglomerates of genera, respectively.

Different Types of Information Used in Bacterial Polyphasic Taxonomy

In principle, all genotypic, phenotypic, and phylogenetic information may be incorporated in polyphasic taxonomy. Genotypic information is derived from the nucleic acids (DNA and RNA) present in the cell, whereas phenotypic information is derived from proteins and their functions, different chemotaxonomic markers, and a wide range of other expressed features (Fig. 1). The number of different molecules which have been applied in taxonomic studies is large, and their applications as markers are manifold. Several of the methods described briefly below (e.g., determinations of the moles percent G+C content and DNA-DNA hybridization studies) became classic and were applied in taxonomic analyses of virtually all bacteria. Others, such as amino acid sequencing, were performed on a limited number of taxa only, because they are laborious, time-consuming, or technically demanding or because they were applicable to only one particular taxon.

Working one's way through lists of methods, it is of primary interest to understand at which level these methods carry information and to realize their technical complexity, i.e., the amount of time and work required. The taxonomic information level of some of these techniques is illustrated in Fig. 2. Obviously, typing methods such as restriction enzyme patterning, multilocus enzyme electrophoresis, and serological analyses are not useful for phylogenetic studies, whereas rRNA or protein sequencing is, in general, not adequate to type large numbers of strains. Chemotaxonomic methods such as fatty acid analysis are fast methods, which allow us to compare and group large numbers of strains in a minimal period, whereas DNA-DNA hybridization studies, for example, will be restricted to a minimal but representative set of strains.

The list of methods given below is not meant to be complete or to contain a description of all of their aspects. It comprises the major categories of taxonomic techniques required to study bacteria at different taxonomic levels and will roughly describe their general concept and applications.

Genotypic Methods

Genotypic methods are those that are directed toward DNA or RNA molecules. Undoubtedly, these methods presently dominate modern taxonomic studies as a consequence of technological progress, but primarily because our present view on classification is that it should reflect the natural relationships as encoded in the DNA. In fact, we are only substantiating our own dogma.

Determination of the DNA base ratio (moles percent G+C). Determination of the moles percent guanosine plus cytosine is one of the classical genotypic methods and is considered part of the standard description of bacterial taxa. Generally, the range observed is not more than 3% within a well-defined species and not more than 10% within a well-defined genus (288). It varies between 24 and 76% in the bacterial world.

DNA-DNA hybridization studies. As mentioned above, the percent DNA-DNA hybridization and the decrease in thermal stability of the hybrid are used to delineate species (343). The percent DNA binding (57) or the DNA-DNA hybridization value or the relative binding ratio (21, 121, 248) is an indirect parameter of the sequence similarity between two entire genomes. It has been established that thermal stabilities decrease from 1 to 2.2% for each 1% of mispairing (13, 287, 306). It is, however, highly debatable whether data which were obtained with short oligonucleotides and experimentally induced mispairing can be extrapolated to entire genomes. At present, it therefore remains impossible to convert a percent DNA-binding or DNA-DNA hybridization value into a percentage of whole-genome sequence similarity.

Different methods have been described: the hydroxyapatite method (21), the optical renaturation rates method (57), and the S1 nuclease method (52, 121) are the most common. The advantage of the optical renaturation rates method is that the DNA needs no label, but it has the inconvenience of not allowing ΔT_m determinations and of being taxonomically insignificant below approximately 30%. The hydroxyapatite method and the two procedures of the S1 nuclease method allow determination of the ΔT_m and have been compared (121). It has been shown that the results obtained by these methods give different relative binding ratios but similar ΔT_m values. The most similar relative binding ratios were obtained by the S1-DE81 procedure and the hydroxyapatite method when hybridized at 75°C (120, 121). These classical techniques, however, need considerable amounts of DNA and are time-consuming. New, quick methods consuming less DNA have been described (93, 152) and are promising to replace the classical methods provided that they are further compared with them under strictly comparable conditions. Indeed, a large number of DNA-DNA hybridization protocols have been described, and it is often not clear whether if hybridizations are performed under optimal, stringent, or suboptimal conditions.

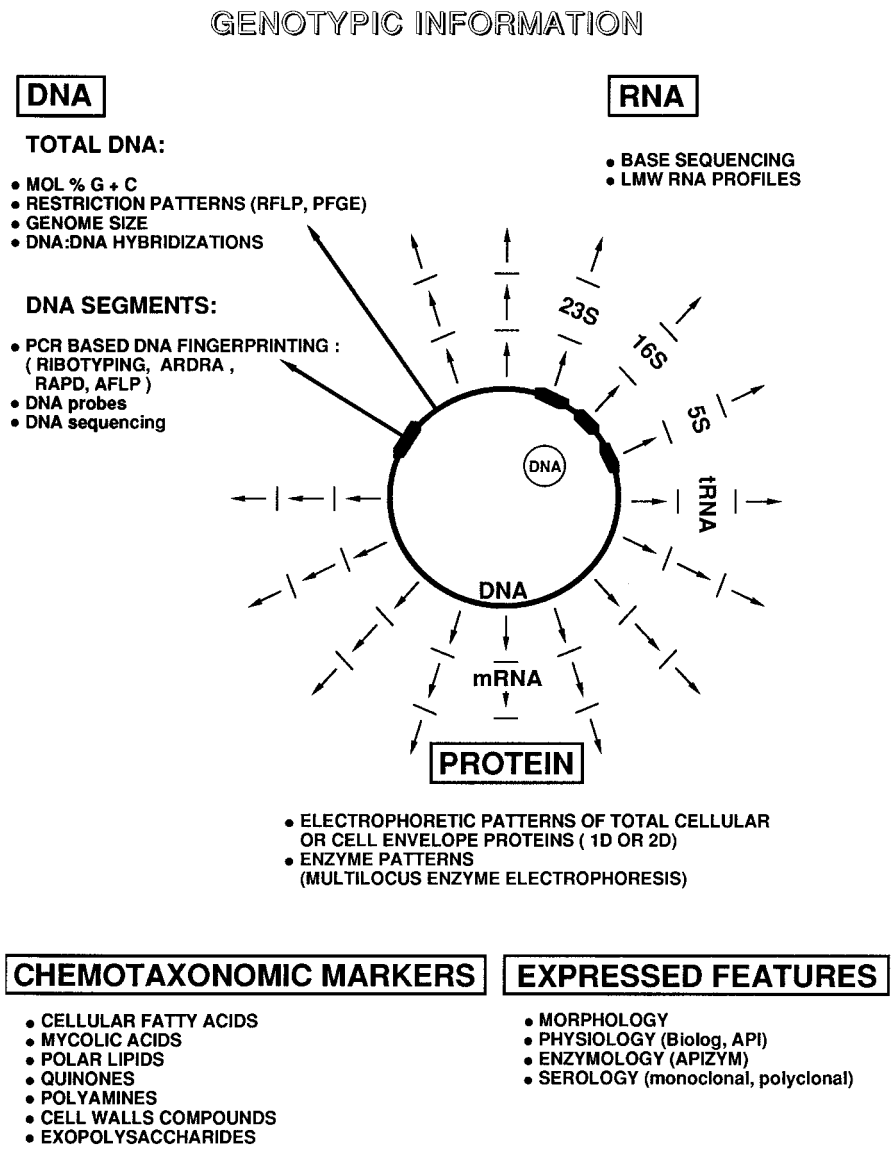

FIG. 1. Schematic overview of various cellular components and techniques used. RFLP, restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; ARDRA, amplified rDNA restriction analysis; RAPD, randomly amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; LMW, low molecular weight; 1D, 2D, one- and two-dimensional, respectively.

The stringency of the reaction is determined by the salt and formamide concentrations and by the temperature and the moles percent G+C of the DNAs used. DNA-DNA hybridizations are often performed under standard conditions that are not necessarily optimal or stringent for all bacterial DNAs. Generally, optimal conditions for hybridizations are preferred, because the optimal temperature curve for hybridization is rather broad (about 5°C). As a rule, renaturation or hybridization under optimal conditions requires a temperature of 22 to 26°C (mean, 24°C) below the melting temperature, measured or calculated at equal salt concentration. The melting temperature (T_m) can be calculated from the salt concentration and the DNA base ratio by using different equations (57, 200). The melting temperature and the optimal hybridization temperature decrease by 0.6°C for each percentage of formamide added to the melting or hybridization mixture (158).

rRNA homology studies. It is now generally accepted that rRNA is the best target for studying phylogenetic relationships because it is present in all bacteria, is functionally constant, and is composed of highly conserved as well as more variable domains (274, 287, 363).

The components of the ribosome (rRNA and ribosomal proteins) have been the subject of different phylogenetic studies for several decades. The gradual development of new molecular techniques enabled the microbiologist to focus on the comparative study of the rRNA molecules. Indirect comparison by either hybridization studies (58, 240) or rRNA cataloging of RNase T_1-resistant oligonucleotides of 16S rRNA (100, 101, 290) have already revealed the natural relationships with and within a number of bacterial lineages (for reviews, see references 56 and 363). Later, sequencing of the rRNA molecules gradually resulted in an rRNA sequence database of 5S

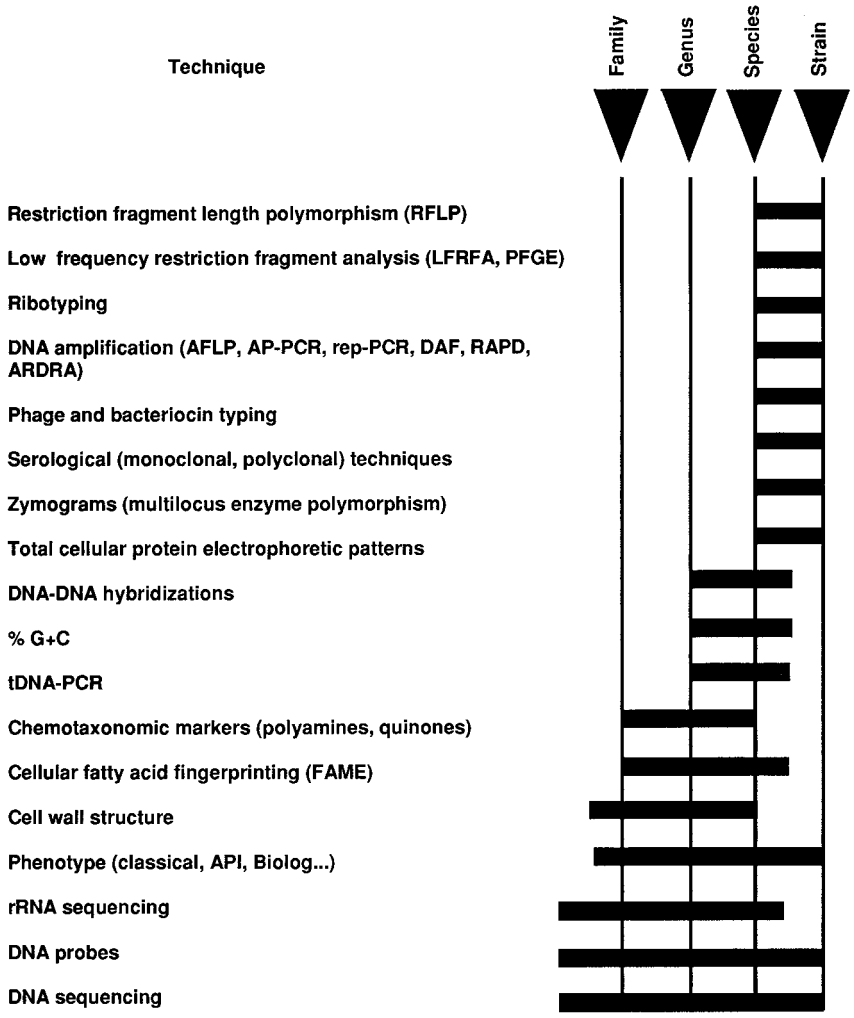

FIG. 2. Taxonomic resolution of some of the currently used techniques. Abbreviations are defined in the legend to Fig. 1.

rRNA (365), which was the first rRNA molecule to be sequenced for numerous bacteria because of its less complex primary and secondary structures. A limited number of 16S rRNA gene sequences became available by direct sequencing after cloning of the genes from the bulk of the DNA (195). Sequencing of 16S rRNA with conserved primers and reverse transcriptase (176) was a very important advance in bacterial phylogeny and resulted in a spectacular increase in 16S rRNA sequences. Nowadays, these techniques have mostly been replaced by direct sequencing of parts or nearly entire 16S or 23S rDNA molecules by using the PCR technique and a selection of appropriate primers. They provide a phylogenetic framework which serves as the backbone for modern microbial taxonomy. The results obtained and the dendrograms constructed with data obtained from the above methods are more or less equivalent, taking into account the specific resolution of each method. However, it is obvious that the larger the conserved elements, the more information they bear and the more reliable the conclusions become. The cataloging method and the DNA-rRNA hybridization experiments have gradually disappeared, although the latter method had the important advantage that multiple strains could easily be included. International databases comprising all published and some unpublished partial or complete sequences have been constructed (66, 226).

DNA-based typing methods. DNA-based typing methods generally refer to techniques which allow us to subdivide species into a number of distinct types. Classically, subtyping of species was performed by means of phenotypic analyses such as biochemical (hence biotyping) or serological (hence serotyping) tests, antibiotic susceptibility patterning, phage or bacteriocin typing, and many others (see below). During the last few years, a battery of DNA-directed typing methods has been developed. Ideally, these techniques are universally applicable (the number of nontypeable strains, if any, is mostly small), they are reproducible and simple to perform, and they are highly discriminatory. Although several of the techniques listed below do not conform to this general description, genotyping has replaced classical typing in many laboratories and will most probably continue to do so (202, 302).

The first-generation DNA-based typing methods included whole-genome restriction fragment analysis and plasmid DNA analysis. In the former, whole-genome DNA is extracted and digested with restriction enzymes. The resulting array of DNA fragments is separated and visualized by agarose gel electrophoresis, and restriction fragment length polymorphisms are

established. The technique has the disadvantage that often very complex patterns of DNA fragments are generated, which are very difficult to compare.

The disadvantages of plasmid analysis are obvious. Strains do not always contain or keep their plasmids, and most strains often belong to only a few types. Restriction fragment analysis of plasmids combines the two techniques and generates more simple banding patterns; it is necessary to establish the identity of plasmids with equal molecular weight. Methods that were elaborated subsequently have reduced the number of DNA fragments compared with the former method and enhanced the reliability and discriminatory power.

The number of DNA fragments can be reduced by selecting restriction enzymes which only rarely cut DNA, recognizing a specific combination of six to eight bases. The technique is referred to as low-frequency restriction fragment analysis. The fragments, however, are too large to be separated by conventional agarose gel electrophoresis. The technique of low-frequency restriction fragment analysis has therefore been dependent on the development of special electrophoretic techniques, generally known as pulsed-field gel electrophoresis, and is now often considered to be the most discriminatory DNA-based typing method (118, 202, 302).

Alternatively, the complex DNA patterns generated after restriction enzyme digestion can be transferred to a membrane and then hybridized with a labeled probe, which allows us to reveal the hybridized fragments. A typical example of one of these developments is the ribotyping method, which uses rRNA as probe (119). Since its initial description, many variants have been presented, but the general principle has remained the same. The rRNA probe may vary in both the labeling technique and sequence. For example, 16S or 23S rRNA or both, with or without the spacer region, or a conserved oligonucleotide part of the rRNA can be used (17).

Also, DNA sequences corresponding to elongation factor Tu, ribosomal protein S12, and flagellar proteins have all been used as probes (116).

The introduction of the PCR methodology into the microbiology laboratory has opened a vast array of applications. Among others, a battery of different typing methods was developed. PCR-based DNA-typing methods attracted much interest because of their universal applicability, simplicity, and rapidity. Different methods in which short arbitrary sequences were used as primers in the PCR assay were described: oligonucleotides of about 20 bases are used in arbitrarily primed PCR (346); oligonucleotides of about 10 bases are used in randomly amplified polymorphic DNA analysis (362); and oligonucleotides of about 5 bases are used in DNA-amplified fingerprinting (28). Alternatively, consensus motifs complementary to fragments of repetitive elements dispersed throughout the genomes of gram-positive or gram-negative bacteria (196, 338) or to tRNA gene fragments (204) may be used as primers. The latter PCR-based method was reported to allow differentiation at the species (347) and infraspecific (276) levels depending on the stringency of the PCR conditions. PCR assays have also been used to amplify the rDNA genes (with or without spacer regions) by means of universal rDNA primers. The polymorphisms between the different rRNA operons generate simple arrays of DNA fragments with different lengths (170).

PCR-based DNA typing was combined with restriction enzyme analysis in the so-called amplified-rDNA restriction analysis method. The PCR product, being 16S or 23S rDNA or parts of both genes with or without the spacer region, is amplified by using universal primers located in the conserved regions of the rRNA genes. The amplicon is subsequently digested with a selected combination of restriction enzymes. In contrast to most other DNA-based methods, amplified-rDNA restriction analysis generates mostly species-specific patterns (122, 155, 174, 258, 331), which is not unexpected considering the conserved character of the rRNA genes.

Another combination of the PCR and restriction enzyme methodologies yielded the AFLP (amplified fragment length polymorphism) technique (372). The basic principle of AFLP is restriction fragment length polymorphism analysis but with a PCR-mediated amplification to select particular DNA fragments from the pool of restriction fragments. AFLP screens for amplified fragment length polymorphisms by selective amplification of restriction fragments. The restriction is performed by using two restriction enzymes, which yield DNA fragments with two different types of sticky ends, combined randomly. To these ends, short oligonucleotides (adapters) are ligated to form templates for the PCR. The selective amplification reaction is performed by using two different primers, containing the same sequence as the adapters but extended to include one or more selective bases next to the restriction site of the primer. Only fragments which completely match the primer sequence are amplified. The amplification process results in an array of about 30 to 40 DNA fragments, some of which are group specific while others are strain specific (153). The technique can therefore be used simultaneously for identification purposes and typing purposes.

Apart from the application of tRNA sequences in the PCR-based typing methods mentioned above, the tRNA gene pool can be used in a so-called low-molecular-weight RNA profiling method (146). These fingerprints comprise the 5S rRNA and the total tRNA pool, which appear on one-dimensional gels as a set of bands belonging to three different classes (148). The rRNA fraction of the profiles allows us to discriminate between some of the major eubacterial groups, while the tRNA fraction reveals more specific taxonomic information (147, 148).

Phenotypic Methods

Phenotypic methods comprise all those that are not directed toward DNA or RNA; therefore, they also include the chemotaxonomic techniques. As the introduction of chemotaxonomy is generally considered one of the essential milestones in the development of modern bacterial classification, it is often treated as a separate unit in taxonomic reviews. The term "chemotaxonomy" refers to the application of analytical methods to collect information on various chemical constituents of the cell to classify bacteria. As for the other phenotypic and the genotypic techniques, some of the chemotaxonomic methods have been widely applied on vast numbers of bacteria whereas others were so specific that their application was restricted to particular taxa.

Classical phenotypic analyses. The classical or traditional phenotypic tests are used in identification schemes in the majority of microbiology laboratories. They constitute the basis for the formal description of taxa, from species and subspecies up to genus and family. While genotypic data are used to allocate taxa on a phylogenetic tree and to draw the major borderlines in classification systems, phenotypic consistency is required to generate useful classification systems and may therefore influence the depth of a hierarchical line (343). The paucity of phenotypic characteristics in particular bacterial groups often causes problems in describing or differentiating taxa. A typical example concerns the genus *Campylobacter* and allied bacteria (see below). For such bacteria, alternative chemotaxonomic or genotypic methods are often required to re-

liably identify strains. In addition, the phenotype of endosymbionts or unculturable bacteria is beyond the reach of our present methods.

The classical phenotypic characteristics of bacteria comprise morphological, physiological, and biochemical features. Individually, many of these characteristics have been shown to be irrelevant as parameters for genetic relatedness, yet as a whole, they provide descriptive information enabling us to recognize taxa. The morphology of a bacterium includes both cellular (shape, endospore, flagella, inclusion bodies, Gram staining) and colonial (color, dimensions, form) characteristics. The physiological and biochemical features include data on growth at different temperatures, pH values, salt concentrations, or atmospheric conditions, growth in the presence of various substances such as antimicrobial agents, and data on the presence or activity of various enzymes, metabolization of compounds, etc. Very often, highly standardized procedures are required to obtain reproducible results within and between laboratories (see, e.g., references 227 and 228).

Numerical analysis. Phenotypic data were the first to be analyzed by means of computer-assisted numerical comparison. In the 1950s, numerical taxonomy arose in parallel with the development of computers (285) and allowed comparison of large numbers of phenotypic traits for large numbers of strains. Data matrices showing the degree of similarity between each pair of strains and cluster analysis resulting in dendrograms revealed a general picture of the phenotypic consistency of a particular group of strains. Obviously, such large numbers of data reflect a considerable amount of genotypic information, and it soon became evident, by comparing the results of such cluster analyses with those of other taxonomic approaches, that analysis of large numbers of phenotypic characteristics was indeed taxonomically relevant.

Automated systems. Miniaturized phenotypic fingerprinting systems have been introduced and may in the future replace classical phenotypic analyses. These systems mostly contain a battery of dehydrated reagents, and addition of a standardized inoculum initiates the reaction (growth, production of enzymatic activity, etc.). The results are interpreted as recommended by the manufacturer and are readily available with a minimal input of time. The outcome of a particular test with a commercial system is sometimes different from that with a classical procedure, but the same is often true for two classical procedures in the same test. Clearly, phenotypic tests must be performed under well-standardized conditions to obtain reproducible results.

Typing methods. Many of the cellular compounds which belong to the bacterial phenotype have been used in typing systems to characterize strains at the infraspecific level. Simple biotyping systems were used which involved a number of tests yielding variable results within species. Serotyping is based on the presence of variability in the antigenic constituents of the cells (142). Structural components such as capsules, cell envelopes, flagella, or fimbriae and intracellular molecules or secretion products such as enzymes and toxins have all been used in serological studies. Antigens may be proteins or carbohydrates and may be thermostable or thermolabile. Different kinds of serological reactions, including simple precipitation or agglutination tests and reactions requiring one or more additional components, such as complement fixation tests, have been described (142).

Many of the described typing techniques are suitable for only some organisms and are performed by only a few reference laboratories (202, 302). As their application in taxonomy is restricted, they will not be discussed here. One of the phenotypic typing methods which is still used for various bacteria is multilocus enzyme electrophoresis. In this technique, native enzymes are electrophoretically separated and stained for enzyme activity and their mobilities are compared. The mobility is an indicator for the existence of multiple polymorphisms of the encoding gene (279). Multilocus enzyme electrophoresis has been extensively used in population genetics, allowing us to establish the overall genetic relatedness of bacterial strains.

Cell wall composition. Determination of the cell wall composition has traditionally been important in gram-positive bacteria. The peptidoglycan type of gram-negative bacteria is rather uniform and provides little information. Cell walls of gram-positive bacteria, in contrast, contain various peptidoglycan types, which may be genus or species specific (273). The procedure is time-consuming, although a rapid screening method has been proposed (273).

Membrane-bound teichoic acid is present in all gram-positive species (7), whereas cell wall-bound teichoic acid is present in only some gram-positive species (168). Teichoic acids can easily be extracted and purified (96) and can be analyzed by gas-liquid chromatography (97, 98).

Cellular fatty acids. A variety of lipids are present in bacterial cells. Polar lipids are the major constituents of the lipid bilayer of bacterial membranes and have been studied frequently for classification and identification purposes. Other types of lipids, such as sphingophospholipids, occur in only a restricted number of taxa and were shown to be valuable within these groups (159). The lipopolysaccharides present in the outer membranes of gram-negative bacteria can be analyzed by gel electrophoresis, giving typical lipopolysaccharide ladder patterns which are interpreted as variants in the O-specific side chains (74, 283). Fatty acids are the major constituents of lipids and lipopolysaccharides and have been used extensively for taxonomic purposes. More than 300 different chemical structures of fatty acids have been identified. The variability in chain length, double-bond position, and substituent groups has proven to be very useful for the characterization of bacterial taxa (298). Mostly, the total cellular fatty acid fraction is extracted, but particular fractions such as the polar lipids have also been analyzed (90). Cellular fatty acid methyl ester content is a stable parameter provided that highly standardized culture conditions are used. The method is cheap and rapid and has reached a high degree of automation.

Isoprenoid quinones. Isoprenoid quinones occur in the cytoplasmic membranes of most prokaryotes and play important roles in electron transport, oxidative phosphorylation, and, possibly, active transport (36, 39). Two major structural groups, the naphthoquinones and the benzoquinones, are distinguished. The former can be further subdivided into two main types, the phylloquinones, which occur less commonly in bacteria, and the menaquinones. The large variability of the side chains (differences in length, saturation, and hydrogenation) can be used to characterize bacteria at different taxonomic levels (39).

Whole-cell protein analysis. The comparison of whole-cell protein patterns obtained by highly standardized sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has proven to be extremely reliable for comparing and grouping large numbers of closely related strains (164, 255, 335). Numerous studies have revealed a correlation between high similarity in whole-cell protein content and DNA-DNA hybridization (47). The use of SDS-PAGE for general identification purposes is hampered by the fact that it yields only discriminative information at or below the species level.

Polyamines. Although the role of polyamines in the bacterial cell is not entirely clear, they seem to be important in bacterial metabolism (299). The observation of their universal character

and quantitative and qualitative variability turned them into a suitable chemotaxonomic marker that can be determined by gas chromatography (368) or high-performance liquid chromatography (see, e.g., references 30 and 271). Depending on the group of organisms studied, polyamine patterning has been used to trace relatedness at and above the genus level and at the species level (25, 124, 278, 370).

Pyrolysis mass spectrometry, Fourier transformation infrared spectroscopy, and UV resonance Raman spectroscopy. Pyrolysis mass spectrometry, Fourier transform infrared spectroscopy, and UV resonance Raman spectroscopy are sophisticated analytical techniques which examine the total chemical composition of bacterial cells. These methods have been used for taxonomic studies of particular groups of bacteria (198).

EVALUATION OF POLYPHASIC TAXONOMY

Polyphasic taxonomy has been applied to many bacterial groups to attain a consensus assessment on the basis of all the available genotypic and phenotypic data. The strengths and weaknesses of the approach are best demonstrated by outlining studies of four bacterial groups with which we have considerable experience.

The genus *Xanthomonas* is an example of a biochemically versatile group, which forms a very tight phylogenetic lineage. The use of a variety of techniques has proven that the previous pathovar notation is an artificial system, and it will be illustrated that the classification and identification based on host specificity were not always correct. The genus *Campylobacter*, on the other hand, represents a biochemically restricted group of bacteria which constitutes an extremely heterogeneous phylogenetic lineage. All members of this group have been studied by large numbers of different methods, illustrating in detail the usefulness of the different techniques in distinguishing taxa at various hierarchical levels. The lactic acid bacteria were chosen to illustrate the problems that occur if settled phenotypic classification schemes do not corroborate phylogenetic insights based on rRNA sequencing. The presumed causes of confusion are the large number of species in, e.g., the genus *Lactobacillus*, which are difficult to discriminate with a limited number of phenotypic tests, and the overreliance on morphological and biochemical characteristics. For the family *Comamonadaceae*, polyphasic analysis led to a transition type of taxonomy in which a compromise was formulated on the basis of the results at hand. It is an example of a family exclusively delineated by using DNA-rRNA hybridization data and in which phenotypic coherence had a major impact on the delineation of genera and species. It represents a biochemically very diverse group of bacteria, which form a tight phylogenetic cluster.

Polyphasic Taxonomy of the Genus *Xanthomonas*

Bacteria belonging to the genus *Xanthomonas* are plant pathogens for at least 124 monocotyledonous and 268 dicotyledonous plant species, on which they cause a variety of disease symptoms including necrosis, gummosis, and vascular or parenchymatous diseases on leaves, fruits, or stems (186). The *Xanthomonas* diseases may cause serious economic losses, e.g., on bean, cassava, citrus fruit, cotton, crucifers, gramineae, poplar, rice, sugarcane, and tomato (138), and identification is crucial.

The classification of the genus *Xanthomonas* as described in *Bergey's Manual of Systematic Bacteriology* (20) underwent a few changes and finally comprised six species: *Xanthomonas albilineans*, *Xanthomonas axonopodis*, *Xanthomonas campestris*, *Xanthomonas fragariae*, *Xanthomonas oryzae*, and *Xanthomonas populi*. *X. campestris* was composed of over 140 pathovars which have a more or less limited host range but which are phenotypically almost indistinguishable. This special-purpose classification was designed to meet the practical needs of plant pathologists and was adopted as a provisional solution until classification was established on more generally accepted principles. The pathovar name is derived from the name of the host plant, although in most cases our knowledge of the host range of strains of a particular pathovar was limited as no extensive host range study had ever been performed. The former *X.* (*Pseudomonas*) *maltophilia*, which is not a plant pathogen, can be differentiated from *Xanthomonas* species and has recently been reclassified as *Stenotrophomonas maltophilia* (241).

TABLE 1. Impact of polyphasic examination of the taxonomy of the genus *Xanthomonas*

Date	Species
1991	*X. albilineans*, *X. axonopodis*, *X. campestris*, *X. fragariae*, *X. oryzae*, *X. populi*, *X. maltophilia*
1995[a]	*X. albilineans*, *X. arboricola*, *X. axonopodis*, *X. bromi*, *X. campestris*, *X. cassavae*, *X. codiae*, *X. cucurbitae*, *X. fragariae*, *X. hortorum*, *X. hyacinthi*, *X. melonis*, *X. oryzae*, *X. pisi*, *X. populi*, *X. sacchari*, *X. theicola*, *X. translucens*, *X. vasicola*, *X. vesicatoria*, *S. maltophilia*

[a] After the introduction of polyphasic examination.

During the last 10 years, an extensive examination of the genus *Xanthomonas* has taken place, using a panoply of methods. These studies have addressed (i) the delineation of the genus *Xanthomonas*, (ii) the species within this genus, (iii) the pathovar system, and (iv) the problem of identification of nonvirulent *Xanthomonas* strains. The application of different genotypic and phenotypic methods (including chemotaxonomic markers) has shed new light on the taxonomy of this plant pathogen and will be summarized in the following paragraphs.

DNA-DNA hybridization studies. Extensive DNA-DNA hybridizations between *Xanthomonas* strains allowed the distinction of 20 DNA hybridization groups (332), of which 4 contain the species *X. albilineans*, *X. fragariae*, *X. populi*, and *X. oryzae*, and 16 are clearly not consistent with the current classification. The latter 16 genomic groups have been described as new species and are composed of one or more former *X. campestris* pathovars or part of them (Table 1). One DNA hybridization group, X9, consisted of *X. axonopodis* and 34 former *X. campestris* pathovars! Extensive DNA-DNA hybridizations not only revealed the natural relationships between *Xanthomonas* strains but also clearly demonstrated that a number of pathovars were composed of two or more unrelated genotypes and could thus not be regarded as biological entities. Table 1 demonstrates the impact of polyphasic examination of the taxonomy of the genus *Xanthomonas*. It might erroneously suggest that polyphasic taxonomy is mainly an activity of "taxonomic splitters," but this would not give credit to the tremendous inputs of polyphasic analyses that have been performed. Within the genus *Xanthomonas*, the major effort was devoted to DNA-DNA hybridizations and has resulted in a complete matrix (332).

16S rRNA sequences. The different *Xanthomonas* species delineated by DNA-DNA hybridizations showed more than 97.8% 16S rDNA sequence similarity and showed 95.2% similarity versus *Stenotrophomonas* (135, 212).

DNA base ratio. The percent G+C values of the genera *Xanthomonas* and *Stenotrophomonas* range between 63 and 70% and between 65 and 68%, respectively.

Numerical analysis of phenotypic features. Van den Mooter and Swings (330) have numerically analyzed 295 phenotypic features determined on 266 *Xanthomonas* and related strains and distinguished the following nine phena. Phenon 1 and phenon 2 comprise *S. maltophilia* and *Xylophilus ampelinus* strains, respectively. These organisms were previously included in *Xanthomonas*, solely on the basis of phenotypic similarity. Phenon 3 contains *X. fragariae*; phenon 4 contains *X. albilineans*; phenon 5 contains *X. axonopodis*; phenon 6 contains *X. populi*; phenon 7 contains *X. oryzae*; phenon 8 contains *X. campestris* pv. graminis; and phenon 9 contains a complex of 189 *X. campestris* strains, representing most pathovars. Only 29 differentiating features were found between the phena.

In spite of the very high phenotypic similarity of *Xanthomonas* species, it was shown that extended analyses of large numbers of strains of separate pathovars could reveal differentiating phenotypic features, e.g., between *X. campestris* pv. manihotis and *X. campestris* pv. cassavae (328), between pathovars from grasses (329), and between *X. oryzae* pv. oryzae and *X. oryzae* pv. oryzicola (337).

Monoclonal antibodies. Monoclonal antibodies have been generated for many *Xanthomonas* species and *X. campestris* pathovars (333), and they allow the identification of large numbers of isolates. Phytopathologists hoped to find a simple way to identify pathovars by this technique. The strains of certain pathovars, e.g., *X. campestris* pv. pelargonii and *X. campestris* pv. begoniae, are indeed each characterized by a single common antigen, but strains of other pathovars, e.g., *X. campestris* pv. campestris, *X. campestris* pv. dieffenbachiae, and *X. campestris* pv. vesicatoria, do not share a single common antigen and can be identified only by a panel of monoclonal antibodies. Pathovars with a broad host range and with demonstrated heterogeneity (e.g., by SDS-PAGE of whole-cell proteins) are serologically complex compared with pathovars with a narrow host range and a demonstrated homogeneity. Monoclonal antibody X1 reacts with all *Xanthomonas* and *Stenotrophomonas* isolates and demonstrates the relatedness between the genera.

Whole-cell protein analysis. Vauterin et al. (334) have applied SDS-PAGE of whole-cell proteins to 307 *Xanthomonas* strains and delineated 19 protein electrophoretic clusters. The most aberrant protein patterns were these of the *S. maltophilia* strains. Separate clusters were found for the species *X. albilineans*, *X. fragariae*, *X. populi*, and *X. axonopodis*. The most important and unexpected result of this study was the demonstration of the heterogeneity of many pathovars, e.g., *X. campestris* pv. vesicatoria and *X. campestris* pv. dieffenbachiae. These protein electrophoretic clusters have been used to select strains for subsequent DNA-DNA hybridizations.

Cellular fatty acid analysis. The cellular fatty acid composition of *Xanthomonas* strains (371) is very complex, as over 65 different fatty acids have been found, and also very characteristic through the occurrence of many branched and hydroxy-branched fatty acids. The fatty acid patterns of *Xanthomonas* and *Stenotrophomonas* species are very similar. Moreover, the two genera share a characteristic set of nine fatty acids. Typical for *S. maltophilia* is the occurrence of the fatty acid 17:0 cyclopropane.

Cluster analysis revealed 31 main clusters, including those for the taxa *X. albilineans*, *X. axonopodis*, *X. fragariae*, *X. populi*, *X. oryzae* pv. oryzae, *X. oryzae* pv. oryzicola, *S. maltophilia* and 24 clusters containing *X. campestris* pathovars. This was also an indication that the latter species consists of several biological entities. A reevaluation of the data generated by Yang et al. (371) has allowed the differentiation of the species defined by Vauterin et al. (332, 336).

Xanthomonadins. The yellow pigment of *Xanthomonas* species is composed of brominated octaenes (4, 295), whereas *Stenotrophomonas* strains have chlorinated hexaenes (156).

Polyamines. Both *Xanthomonas* and *Stenotrophomonas* species were characterized by the occurrence of spermidine and low quantities of spermine, but *Stenotrophomonas* species characteristically contained considerable quantities of cadaverine (370).

Conclusions. The recent developments in *Xanthomonas* taxonomy reflect the conflicting interests of a general-purpose and a special-purpose classification, with the first being based on real biological units that are defined by polyphasic taxonomy and the second being defined only by phytopathogenicity.

The genera *Xanthomonas* and *Stenotrophomonas* are phenotypically and genotypically highly related, with the former probably being a recently evolved branch, specializing as a plant pathogen, from a ubiquitously occurring *Stenotrophomonas*-like ancestor. Whether *Xanthomonas* and *Stenotrophomonas* are two separate genera or should be retained in the single genus *Xanthomonas* might be a mere reflection of splitting or lumping opinions of taxonomists.

Polyphasic Taxonomy of the Genus *Campylobacter*

The genera *Campylobacter* and *Arcobacter* form a family of gram-negative, nonsaccharolytic bacteria with microaerobic growth requirements and a low G+C content, the *Campylobacteraceae* (317). As such, this definition is in full agreement with the original criteria used by Sebald and Véron (277) to separate a number of *Vibrio* species from the genuine vibrios and to include them in the newly created genus *Campylobacter*. Members of the family *Campylobacteraceae* are encountered mainly as commensals or parasites in humans and domestic animals. The taxonomic history of these bacteria has been dominated by their biochemical inertness. Classical phenotypic tests routinely used for the identification of clinically significant bacteria often yield negative results or yield variable results within species. This lack of differential characteristics led to the widespread use of vernacular names for many isolates, e.g., gastric *Campylobacter*-like organisms or urease-producing thermophilic campylobacters (terms reflecting the unusual isolation sources or aberrant phenotypic characteristics of the strains). Over a period of about 30 years, these groups were detected, described, and identified as different biotypes of existing species or as new species. The genus *Campylobacter* became a deposit for a wide assemblage of taxa characterized by a minimal set of phenotypic characteristics including the microaerobic growth requirements and a nonsaccharolytic metabolism.

The situation improved when phylogenetic studies revealed a considerable genotypic heterogeneity among these species, and three major natural clusters (rRNA homology groups) were recognized (303, 321). *Campylobacter* was separated into three genera, and a revised genus description was given. New names were proposed for the remaining two natural clusters, *Arcobacter* and *Helicobacter* (113, 321). At present, the genus *Campylobacter* comprises 15 species with *Campylobacter fetus* as the type species (2, 91, 293, 316, 321), *Arcobacter* comprises 4 species with *Arcobacter nitrofigilis* as the type species (326), and *Helicobacter* comprises 12 species with *Helicobacter pylori* as the type species (22, 78, 85, 100, 104, 184, 244, 294, 321). Table 2 lists the members of the genus *Campylobacter* and related bacteria and the modifications which resulted mainly from a polyphasic taxonomic study (321).

TABLE 2. The genus *Campylobacter* and allied bacteria

Date	Genus	Species
1989–1991[a]	*Campylobacter*	*C. cinaedi*, *C. coli*, *C. concisus*, *C. cryaerophila*, *C. fennelliae*, *C. fetus* (type species), *C. hyointestinalis*, *C. jejuni*, *C. lari*, *C. mucosalis*, *C. mustelae*, *C. nitrofigilis*, *C. pylori*, *C. sputorum*, *C. upsaliensis*
	Wolinella	*W. succinogenes* (type species), *W. curva*, *W. recta*
	Bacteroides[b]	*B. gracilis*, *B. ureolyticus*
	Others	About 10 groups of *Campylobacter*-like organisms
1991–1995[c]	*Campylobacter*	*C. helveticus*, *C. hyoilei*, *C. showae*
	Arcobacter	*A. butzleri*, *A. skirrowii*
	Helicobacter	*H. acinonyx*, *H. bilis*, *H. canis*, *H. felis*, *H. hepaticus*, *H. muridarum*, *H. nemestrinae*, *H. pametensis*
July 1995[d]	*Campylobacter*	*C. coli*, *C. concisus*, *C. curvus*, *C. fetus*, *C. gracilis*, *C. helveticus*, *C. hyoilei*, *C. hyointestinalis*, *C. jejuni*, *C. lari*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum*, *C. upsaliensis*
	Arcobacter	*A. nitrofigilis* (type species), *A. cryaerophilus*, *A. butzleri*, *A. skirrowii*
	Helicobacter	*H. acinonyx*, *H. bilis*, *H. canis*, *H. cinaedi*, *H. felis*, *H. fennelliae*, *H. hepaticus*, *H. muridarum*, *H. mustelae*, *H. nemestrinae*, *H. pametensis*, *H. pylori* (type species)
	Wolinella	*W. succinogenes*
	Bacteroides[b]	*B. ureolyticus*

[a] Situation before taxonomic revisions.
[b] Generically misclassified *Bacteroides* species.
[c] New species and genera since 1991.
[d] Present situation.

Below, several techniques and parameters used in taxonomic studies of campylobacters and related bacteria are discussed.

rRNA homology studies. Sequence comparison of 16S rRNA has been used primarily to unravel the taxonomic structure and relationships of *Campylobacter* and affiliated genera (177, 243, 263, 303). As described above, rRNA sequence homology studies allowed us to divide the genus *Campylobacter* into three distinct groups, each corresponding to a separate genus. Within each of the three major groups, the genotypic heterogeneity was considerable, with sequence differences of up to 9.4% in the genus *Campylobacter*, 5.7% in *Arcobacter*, and 8.6% in *Helicobacter* (78, 91). The putative anaerobes *Wolinella recta*, *Wolinella curva*, *Bacteroides gracilis*, and *Bacteroides ureolyticus* belonged to the same rRNA homology group as the genuine campylobacters (the homology group comprising the type species), whereas *Wolinella succinogenes* appeared to be a close neighbor of the *Helicobacter* cluster. Subsequent physiological studies revealed that none of these bacteria were genuine anaerobes (129, 130). Only *B. ureolyticus* and *W. succinogenes* were clearly differentiated from their neighbors in their respective rRNA homology groups. The others could not be separated from campylobacters on genotypic or on phenotypic grounds and were therefore reclassified as *Campylobacter* species (316, 321). *W. succinogenes* was originally separated from *H. pylori* and *Helicobacter mustelae* (the only two *Helicobacter* species at that time) on the basis of differences in ultrastructure, morphology, cellular fatty acid and respiratory quinone components, growth characteristics, and enzyme capabilities (113). However, since then, the number of *Helicobacter* species has increased drastically, and most of these features have not been examined for the recently described species. Finally, in spite of an extensive knowledge of its genotypic and phenotypic characteristics, the classification of *B. ureolyticus* remains unsettled (316).

The application of sequence comparison for studying the intrageneric structure of each of these genera has been hindered by the instability of the branching levels between closely related bacteria (i.e., within the genera). Bootstrap analysis revealed that within the genus *Campylobacter*, several clades of species are stable and therefore their linkage can be considered significant (316). However, other clades have bootstrap values below 55%, and thus the exact branching sequence is not significant. For these species, the branching order easily shifts upon changing the number of taxa included, changing the outgroups, or choosing a different set of relatives (243, 316). Partial 23S rRNA sequences (about 800 bases) have been determined for a number of *Campylobacter* species. The general topology of phylogenetic trees derived from these partial sequences is in overall agreement with that of trees based on complete 16S rRNA cistrons (313).

Sequence information derived from 16S or 23S rRNA cistrons has been used successfully to design a battery of species-, group-, or genus-specific primers and probes (11, 12, 78, 92, 215, 262, 312, 348). Applied in a PCR assay, these primers and probes offer valuable alternatives for the identification of these bacteria.

Most of the *Campylobacter*, *Arcobacter*, and *Helicobacter* species have been included in DNA-rRNA hybridization analyses as well (315, 321, 325). Again, the general topology of the phylogenetic tree confirmed the one based on 16S rRNA sequence data (303, 321). A number of intrageneric relationships were revealed: a close association was found between *C. fetus* and *C. hyointestinalis*; between *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*; and between *C. concisus* and *C. mucosalis*. The former two clusters were also found to be significant after bootstrap analysis of the 16S rRNA sequences (316).

DNA-DNA hybridization studies. Most DNA-DNA hybridization studies were performed before the phylogenetic relationships of campylobacters were established by means of rRNA-directed studies and focused on the species known since the early 1980s (15, 16, 32, 91, 134, 140, 182, 236, 264–266, 270, 307, 308, 319, 323, 326). Significant DNA hybridization was found between *C. fetus* and *C. hyointestinalis* (264, 307); between *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*; (32, 134, 140, 264, 270, 308); and between *C. rectus* and *C. showae* (91). Roop et al. (265, 266) also demonstrated that the former *C. sputorum* subsp. *mucosalis* (180) does not belong to *C. sputorum* and that *C. sputorum* subsp. *sputorum*, *C. sputorum* subsp. *bubulus*, and "*Campylobacter fecalis*" should be considered biovars of a single species, *C. sputorum*. These data were confirmed by Chevrier et al. (32). DNA-DNA hybridizations between all other *Campylobacter* species yielded nonsignificant hybridization val-

ues. The precise level of DNA-DNA binding is difficult to compare because of the lack of correlation when different hybridization techniques are used (see below).

DNA base ratio. The percent G+C values of *Campylobacter*, *Arcobacter*, and *Helicobacter* species range between 30 and 46%, between 27 and 31%, and between 35 and 44%, respectively.

Classical phenotypic characteristics. The cellular morphology of campylobacters and their relatives is extremely diverse. A slender spiral ("corkscrew-like") morphology is generally considered typical. However, such cells, which are most often found in the group of the so-called thermophilic campylobacters (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*), readily lose this morphology upon aging and cells incubated longer than 24 h will transform into bent rods or straight rods and finally may become virtually completely coccoid. Again, cells of other species such as *C. fetus* are spiral yet not slender, and huge spiral or threadlike cells (up to 20 μm long) may be present in old cultures. Cells of other species are predominantly S-shaped rods, bent rods, or even straight rods.

In addition, the diversity in flagellation types is striking as well. Most campylobacters are characterized by a single polar or bipolar flagellum. Sporadic nonmotile cells occur naturally. *C. showae*, however, has polar bundles of two to five flagella, whereas *C. gracilis* (formerly *B. gracilis*) is aflagellate. An even larger diversity is found within the genus *Helicobacter*, in which single flagella, bipolar flagella, and lateral flagella are found in different species. The number of flagella and the flagellation type are obviously not a relevant taxonomic parameter in this group of bacteria. Conversely, the structure of the flagellum appears to be significant, because all taxa of the *Campylobacter* and *Arcobacter* rRNA homology groups have unsheathed flagella whereas a flagellar sheath is present in all *Helicobacter* species (128, 321).

The problems inherent in the identification of campylobacters and their relatives by means of classical phenotypic tests have been mentioned above and have been the subject of several studies (23, 78, 87, 88, 141, 145, 209, 249, 264, 366). They will not be discussed here. Recently, On and Holmes (229) performed a most comprehensive study: they examined 67 characteristics for 347 strains representing all present species within this phylogenetic lineage and subjected the data to numerical comparison. Their data indicated that there was considerable correspondence between the grouping obtained and previously determined genomic relationships and that most strains were accurately identified to the species or subspecies level.

Respiratory quinone components. The study of the respiratory quinone components of campylobacters was of major importance for the revision of their classification. The respiratory chain of campylobacters, unlike most gram-negative bacteria, comprises only menaquinones (37, 38, 114, 217, 218, 316). All species belonging to the *Campylobacter* rRNA homology group, including the former *W. recta*, *W. curva*, *B. gracilis*, and *B. ureolyticus*, contain menaquinone-6 and the so-called thermoplasmaquinon (a methyl-substituted menaquinone-6) as major components. Species belonging to the *Arcobacter* rRNA homology group, the closest phylogenetic neighbor of *Campylobacter* species, are characterized by menaquinone-6 and a second menaquinone, whose structure has not yet been unravelled. The same situation occurs in the *Wolinella-Helicobacter* group, as *Wolinella* species are characterized by menaquinone-6 and the thermoplasmaquinon whereas *Helicobacter* species contain menaquinone-6 and the above-mentioned quinone with the unknown structure.

Cellular fatty acid analysis. Several authors studied the suitability of cellular fatty acid analysis for the differentiation and identification of campylobacters (18, 44, 53, 115, 175, 181, 217, 342). Lambert et al. (175) and Goodwin et al. (115) used similar procedures and performed the most comprehensive studies. They included nearly all known *Campylobacter* taxa and subdivided them into so-called gas-liquid chromatography groups. Several of these gas-liquid chromatography groups consisted of more than one species, and several species were present in different gas-liquid chromatography groups. Classical phenotypic tests were required to identify some strains at the species level. Differences in the cellular fatty acid components were used as one of the criteria for including the former *C. pylori* and *C. mustelae* not in the genus *Wolinella* but in a newly created genus, *Helicobacter* (113). At present, these differences can no longer be considered genus specific, because two additional *Helicobacter* species, *H. cinaedi* and *H. fennelliae*, again had different cellular fatty acid patterns (115) whereas none of the recently described new species has been examined. Clearly, this technique is not efficient for the identification of *Campylobacter* species and a comprehensive study on the various *Helicobacter* species should be performed before general conclusions for this genus can be drawn. In contrast, a detailed study of the genus *Arcobacter* revealed quantitative or qualitative differences (or both) between most of its members (326).

Protein analysis. PAGE of proteins has often been used successfully to classify phenotypically aberrant campylobacters. Since the early 1970s, this technique has been widely applied for the differentiation and identification of campylobacters, arcobacters, and helicobacters. Acid- plus phenol-soluble proteins (131, 216), water-soluble proteins (95, 216, 225), and whole-cell proteins (48–50, 91, 99, 117, 206, 232–234, 237, 246, 293, 294, 301, 316, 318–320, 323–326) are separated in polyacrylamide gels and stained and may be subjected to computer-assisted numerical comparison. The latter is a prerequisite for comparing large numbers of strains. As with other bacteria, strains with highly similar protein patterns share high DNA hybridization values and therefore belong to the same species (47, 235). This correspondence between high correlation coefficients of protein patterns and high DNA hybridization values has been illustrated for several *Campylobacter* and *Arcobacter* species (319, 323, 326).

However, the interpretation of protein pattern similarity regularly requires considerable expertise. In a numerical analysis, variation in the number or molecular weight of one or a few dense protein bands may cause strains of a single species to form distinct clusters. Only a thorough visual examination of the protein electrophoretic traces of strains from each cluster will reveal the reason for such subdivisions. The part of the protein profile with these variable dense bands is situated mostly in the 39,000- to 50,000-molecular-weight range, and it may be necessary to omit this region from the numerical analysis to obtain clusters of closely related strains (47, 318, 319, 323).

Interestingly, whole-cell protein patterns of campylobacters are stable regardless of the time and methods of preservation, growth conditions, and age of the cells (99, 131, 324). Within other bacterial groups, growth medium and growth conditions may considerably influence the protein profile (151, 163). This stability may be explained by the limited nutritional capabilities of the campylobacters.

Conclusion. Various taxonomic parameters of campylobacters and allied bacteria were studied, and a huge amount of data was collected. The taxonomic structure of the entire group is thus well understood. The genera *Campylobacter*, *Arcobacter*, and *Helicobacter* were separated primarily because of

the large genotypic divergence between the three groups (321). This subdivision was supported by chemotaxonomic and ultrastructural characteristics. The large differences in 16S rRNA sequences between and within these genera may indicate that they represent old evolutionary branches in which substantial genetic drift occurred. The enormous range in DNA base ratio which is found in the genus *Campylobacter* is an additional support for this hypothesis.

At the species level, the congruence between the whole-cell protein pattern similarity and DNA-DNA hybridization was clearly established, and the former technique was successfully used to identify phenotypically aberrant strains. New *Campylobacter* and *Helicobacter* species have been emerging rapidly, and although they are mostly well characterized and their phylogenetic position has been clearly established, their identification by means of classical phenotypic tests is cumbersome. Protein electrophoresis offered a valuable alternative for species identification. As mentioned above, the suitability of cellular fatty acid analysis for the differentiation of these species is genus dependent; poor discriminatory power was found in *Campylobacter* species, whereas most *Arcobacter* species were easily differentiated. Helicobacters were only partly examined.

The total number of taxa within this lineage of the *Proteobacteria* now approaches 50. Many of these can be differentiated only by a single or a few classical phenotypic tests or, alternatively, by using a large number of phenotypic tests (229). Molecular diagnostic methods with DNA probes or specific PCR assays or, alternatively, a chemotaxonomic method such as whole-cell protein analysis may therefore become essential to reliably identify unusual or atypical campylobacters pending the discovery of additional phenotypic tests.

Polyphasic Taxonomy of Lactic Acid Bacteria

Lactic acid bacteria are gram-positive, non-spore-forming cocci, coccobacilli, or rods with a DNA base composition of less than 50 mol% G+C. They generally lack catalase, although pseudocatalase was detected in cultures grown at a low sugar concentration. They need a fermentable carbohydrate for growth; glucose is converted mainly to lactic acid (homofermentatives) or to lactic acid, CO_2, and ethanol or acetic acid or both (heterofermentatives). However, this definition generally covers more taxa than are normally covered by the name "lactic acid bacteria." It is mainly their importance in the fermentation of food and feed products (meat, vegetables, fruits, beverages, dairy products, and silage) which delineates the group of lactic acid bacteria. Therefore, lactic acid bacteria are generally restricted to the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. Representatives of the genera *Aerococcus*, *Alloiococcus*, *Atopobium*, *Dolosigranulum*, *Gemella*, *Globicatella*, *Helcococcus*, *Melissococcus*, and *Saccharococcus* are generally not considered to belong to the lactic acid bacteria, although they might, to some extent, meet the definition given above. Because of its use in food and its probiotic role in the human intestinal tract, the genus *Bifidobacterium* is often listed along with the lactic acid bacteria sensu stricto, although phylogenetically it belongs to the *Actinomyces* subdivision of the gram-positive bacteria (comprising also *Atopobium*, *Brevibacterium*, *Propionibacterium*, and the microbacteria) and therefore is only quite distantly related to the genuine lactic acid bacteria, which belong to the *Clostridium* subdivision of the gram-positive bacteria.

In food, lactic acid bacteria contribute to the taste and texture of fermented products and inhibit the growth of food-spoiling bacteria by the production of growth-inhibiting substances (bacteriocins) and the production of large amounts of lactic acid. However, lactic acid bacteria are also known to be involved in spoilage of, e.g., beer and wine and to occur in the respiratory, intestinal, and genital tracts of humans and animals, in sewage, and in plant materials. Many lactic acid bacteria are known to have a positive impact on human and animal health (109). Some pathogenic species are found among, e.g., the streptococci.

Given the very large number of species described, only the most notable species of the lactobacilli, with some related species of *Leuconostoc* and *Pediococcus*, will be highlighted here. Furthermore, it is not our purpose to list the wide variety of techniques used in the study of the taxonomy of these organisms; instead, we will focus on those techniques which have directed some of the major taxonomic changes within the lactobacilli. Table 3 presents a subdivision of these lactobacilli according to their fermentation type and phylogenetic assignment.

Phylogenetic analysis based on rRNA homology. At present, the genus *Lactobacillus* comprises 56 species, 5 of which contain at least two subspecies. During the last several years, 9 *Lactobacillus* species were transferred to other *Lactobacillus* species and at least 14 species were transferred to new or already existing genera (Table 3). These taxonomic reassignments were based almost exclusively on the results of rRNA sequencing or DNA-rRNA hybridizations. The large number of nomenclatural revisions is a striking indication of the discrepancies between the results obtained by former traditional phenotypic tests (classical taxonomy) and the present phylogenetic insights obtained by rRNA sequencing (molecular taxonomy).

In a recent overview of the phylogeny of lactic acid bacteria (275), a phylogenetic tree was constructed by using distance matrix, parsimony, and maximum-likelihood analyses. The major phylogenetic groups of lactic acid bacteria sensu stricto, which are largely in agreement with data published by Collins et al. (41), are described. The lactobacilli can be subdivided into three major groups: the *Lactobacillus delbrueckii* group; the *Lactobacillus casei-Pediococcus* group; and the *Leuconostoc* group.

The percent homologies of 16S rRNA within the two major lactobacillus groups vary from 90.8 to 99.3% (*L. delbrueckii* group) and 90.3 to 99.0% (*L. casei-Pediococcus* group) (41). Within the *Leuconostoc* group, the position of *Oenococcus oeni* is very interesting. Extremely low homology values of 85.9 to 91.5% and 85.9 to 86.8% were registered between this organism and the other species in the same group (201). This specific phylogenetic structure has been discussed as a case of a rapidly evolving organism (369) and was one of the arguments in removing the species from the genus *Leuconostoc* into the genus *Oenococcus* (79).

(i) *L. delbrueckii* group. The *L. delbrueckii* group contains *L. delbrueckii* (the type species of the genus *Lactobacillus*), the seven species of the *L. acidophilus* group (see below), *L. acetotolerans*, *L. hamsteri*, *L. jensenii*, *L. kefiranofaciens*, and, peripherally, *L. amylophilus.*

L. delbrueckii contains three subspecies which cannot be discriminated by rRNA sequence analysis.

(ii) *L. casei-Pediococcus* group. The second phylogenetic group of the lactobacilli, the *L. casei-Pediococcus* group (41, 275), with 37 *Lactobacillus* species and 5 *Pediococcus* species, outnumbers the other groups. Four of the pediococcal species (*Pediococcus acidilactici*, *P. damnosus*, *P. parvulus*, and *P. pentosaceus*) form a separate clade. *P. dextrinicus* is more closely related to *L. coryniformis* and *L. bifermentans* than to the other

TABLE 3. Subdivision of the lactobacilli according to their phenotype and phylogenetic assignment[a]

Phylogenetic group	Species in fermentation group:		
	A (obligately homofermentative)	B (facultatively heterofermentative)	C (obligately heterofermentative)
a (*L. delbrueckii* group)	*L. acidophilus*, *L. amylophilus*, *L. amylovorus*, *L. crispatus*, *L. delbrueckii* subsp. *bulgaricus* ("*L. bulgaricus*"), subsp. *delbrueckii*, subsp. *lactis* ("*L. lactis*"), *L. gallinarum*, *L. gasseri*, *L. helveticus* ("*L. jugurti*"), *L. jensenii*, *L. johnsonii*, *L. kefiranofaciens*, *L. kefirgranum*[a]	*L. acetotolerans*, *L. hamsteri*	
b (*L. casei-Pediococcus* group)	*L. aviarius* subsp. *aviarius*, subsp. *araffinosus*, *L. farciminis*, *L. ruminis*, *L. mali* ("*L. yamanashiensis*"), *L. salivarius* subsp. *salicinus*, subsp. *salivarius*, *L. sharpae*, *Pediococcus damnosus*, *Pediococcus dextrinicum*, *Pediococcus parvulus*	*L. agilis*, *L. alimentarius*, *L. casei*, *L. bifermentans*, *L. coryniformis*, subsp. *coryniformis*, subsp. *torquens*, *L. curvatus*, *L. graminis*, *L. homohiochii*, *L. intestinalis*, *L. murinus* ("*L. animalis*"), *L. paracasei* subsp. *paracasei*, subsp. *tolerans*, *L. pentosus*, *L. plantarum*, *L. rhamnosus*, *L. sake* ("*L. bavaricus*") *Pediococcus acidilactici*, *Pediococcus pentosaceus*	*L. brevis*, *L. buchneri*, *L. collinoides*, *L. fermentum* ("*L. cellobiosus*"), *L. fructivorans* ("*L. trichodes*"), *L. hilgardii* ("*L. vermiforme*"), *L. kefir*, *L. malefermentans*, *L. oris*, *L. parabuchneri*, *L. parakefir*,[a] *L. pontis*, *L. reuteri*, *L. suebicus*, *L. sanfrancisco*, *L. vaccinostercus*, *L. vaginalis*
c (*Leuconostoc* group)			*L. fructosus*, *W. confusus* ("*L. confusus*"), *W. halotolerans* ("*L. halotolerans*"), *W. kandleri* ("*L. kandleri*"), *W. minor* ("*L. minor*"), *W. viridescens* ("*L. viridescens*"), *W. hellenica*, *W. paramesenteroides* ("*Leuconostoc paramesenteroides*"), *Leuconostoc amelibiosum*, *Leuconostoc argentinum*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc gelidum*, *Leuconostoc carnosum*, *Leuconostoc fallax*, *O. oeni*
Other lactobacilli[b]	*L. catenaformis*, *L. vitulinus*, *L. rogosae*,[c] *Atopobium minutum* ("*L. minutus*"), *Atopobium rimae* ("*L. rimae*"), *Atopobium uli* ("*L. uli*"), *Carnobacterium divergens* ("*L. divergens*") ("*L. carnis*"), *Carnobacterium piscicola* ("*L. piscicola*") ("*L. maltaromicus*"), *Lactococcus lactis* ("*L. hordniae*") ("*L. xylosus*")		

[a] Data from reference 126. *L. kefirgranum* and *L. parakefir* have not been included in 16S rRNA sequence analysis.
[b] Species in this group are no longer considered members of the genus *Lactobacillus* and are assigned to various fermentation groups.
[c] *L. rogosae* was described to have a DNA base composition of 59 mol% G+C, which is outside the range defined for the genus *Lactobacillus*; no strains corresponding to the original description are presently available.

Pediococcus species. The position of the latter lactobacillus is particularly interesting, since *L. bifermentans* shows, depending on the pH, an aberrant type of fermentation (161, 247).

The *L. casei-Pediococcus* group contains a number of well-known lactobacilli including the poorly characterized species *L. plantarum*, *L. paracasei*, which is found in many habitats (dairy products, wine, silage, sewage, and the human intestinal tract and mouth), and also *L. sake*, *L. curvatus*, and *L. bavaricus*, which are used in many food fermentations.

(iii) *Leuconostoc* group. The third phylogenetic group within the lactobacilli, the *Leuconostoc* group (41), comprises two subgroups. A first subgroup is the so-called *paramesenteroides* cluster containing the former species *Lactobacillus confusus*, *L. halotolerans*, *L. kandleri*, *L. minor*, *L. viridescens*, and *Leuconostoc paramesenteroides*. The latter species is quite far removed from the other leuconostocs, *Lactobacillus fructosus*, and *O. oeni* (the former *Leuconostoc oenos*), which together form the second subgroup within the *Leuconostoc* group (275). More recently, the *paramesenteroides* group (42) was assigned to the new genus *Weissella*, comprising the species *Weissella confusus*, *W. halotolerans*, *W. kandleri*, *W. minor*, *W. paramesenteroides*, *W. viridescens*, and a new species, *W. hellenica* for isolates from fermented sausage (42).

(iv) Other lactobacilli. Finally, rRNA sequence analyses revealed a considerable number of misnamed lactobacilli, related to the clostridia and *Erysipelothrix* (*Lactobacillus catenaformis*, *L. vitulinus*), to the actinomycete branch of the gram-positive bacteria (*L. minutus*, *L. rimae*, and *L. uli*, all transferred to the genus *Atopobium* by Collins and Wallbanks [43]), or to the lactococci (*L. xylosus* and *L. hordniae*, both transferred to *Lactococcus lactis* [165]).

Delineation of *Lactobacillus* species by traditional phenotypic tests. The genus *Lactobacillus* was proposed by Beijerinck in 1901 (14). Since then, many species have been described but either not validated or, as mentioned above, shown to be homologous to existing species. Examination of the presence of fructose-1,6-bisphosphate aldolase resulted in the subdivision of these bacteria into three physiological groups: (i) the obligately homofermentative lactobacilli, (ii) the facultatively heterofermentative species, and (iii) the obligately heterofermentative species which lack fructose-1,6-bisphosphate aldolase. Although correct classification and identification of lactic acid bacteria are difficult without the support of modern genotypic techniques (see above), the large number of species renders an exclusively genotypic approach quite cumbersome. Therefore, fermentation reactions (mostly examined via commercially available test systems) remain important as tools for identification and classification.

The subdivision of lactobacilli into fermentation groups was maintained with some major modifications until the late 1970s, (260, 261, 281) and is still used today. To cope with the increasing number of newly described species, the fermentation groups were consecutively redefined in 1986 by Kandler and Weiss (162) and by Hammes et al. (127). In an attempt to match the phenotypic division of the lactobacilli with the phylogenetic data obtained from rRNA sequencing, the subdivision described below was proposed by Hammes and Vogel (126). In this subdivision, the capital letters A, B, and C, refer to fermentation types and the lowercase letters a, b, and c, refer in each subdivision to the three phylogenetic subgroups (the *L. delbrueckii* group, the *L. casei-Pediococcus* group, and the *Leuconostoc* group, respectively).

(i) Obligately homofermentative lactobacilli (group A). Obligately homofermentative lactobacilli degrade hexoses almost exclusively to lactic acid by the Embden-Meyerhof pathway, cannot use pentoses or gluconate, and include the thermobacteria and a considerable number of more recently described species. The phenotypic differentiation of the obligately homofermentative lactobacilli is given by Hammes and Vogel (126). The obligately homofermentative lactobacilli are allocated to two phylogenetic subgroups, the *L. delbrueckii* subgroup (group Aa) and the *L. casei-Pediococcus* subgroup (group Ab). Besides *L. delbrueckii* and *L. jensenii*, subgroup Aa contains the industrially important assembly of species known as the *L. acidophilus* group (157). Despite its industrial and medical importance (136, 160, 210, 259), the taxonomy of *L. acidophilus* remained confusing for a long time (106, 133, 214, 231, 280). A first indication of its heterogeneity was obtained from serological data (86). Later, electrophoretic analysis of soluble cellular proteins or lactate dehydrogenases, detailed cell wall studies, and determination of the moles percent G+C content of the DNA confirmed the presence of a number of genotypic groups, each deserving a separate species status. Finally, DNA-DNA hybridization studies and standardized SDS-PAGE of whole-cell proteins (see below) have been necessary to actually delineate the species present in this pool of phenotypically very similar organisms. At present, these species still cannot be reliably differentiated by simple phenotypic tests (105).

Some presumed obligately homofermentative lactobacilli of the phylogenetic group of *L. delbrueckii* (*L. acetotolerans*, *L. amylophilus*, *L. hamsteri*, and *L. kefiranofaciens*) can ferment pentoses and were therefore classified as facultatively heterofermentative lactobacilli in group B (as subgroup Ba [126]). It has been suggested that the group A species may have evolved from metabolically more versatile facultative heterofermenters, e.g., by the loss of phosphoketolase activity (126).

(ii) Facultatively heterofermentative lactobacilli (group B). Facultatively heterofermentative lactobacilli ferment hexoses almost exclusively to lactic acid by the Embden-Meyerhof pathway or to lactic acid, acetic acid, ethanol, and formic acid under glucose limitation. Pentoses are fermented to lactic acid and acetic acid via an inducible pentose phosphoketolase; the group includes the former streptobacteria and many newly described species.

Again, two subgroups of species are distinguished: group Ba (facultatively heterofermentative lactobacilli from the *L. delbrueckii* rRNA branch) comprises *L. acetotolerans* and *L. hamsteri* (both regarded as obligate homofermenters until recently [126]), and group Bb (facultatively heterofermentative lactobacilli from the *L. casei-Pediococcus* branch) harbors 15 *Lactobacillus* species (126).

(iii) Obligately heterofermentative lactobacilli (group C). The obligately heterofermentative lactobacilli ferment hexoses to lactic acid, acetic acid, ethanol, and CO_2 via the phosphogluconate pathway. Pentoses are fermented to lactic acid and acetic acid. In general, a pentose phosphoketolase is involved in both pathways. According to Hammes and Vogel (126), a total of 22 *Lactobacillus* species are spread over two phylogenetic branches: 16 species have been assigned to group Cb (obligately heterofermentative lactobacilli from the *L. casei-Pediococcus* branch), and 6 have been assigned to group Cc (obligately heterofermentative lactobacilli from the *Leuconostoc* branch), 5 of which have recently been transferred to the genus *Weissella* (42). As for the lactobacilli of group A, the species of group Cb may have been derived from the metabolically more versatile group Bb species by the loss of the aldolase and triose-phosphate isomerase of the Embden-Meyerhof pathway.

Delineation of *Lactobacillus* species by DNA-DNA hybridization studies. As with *Campylobacter* species, most DNA-DNA hybridization studies were performed before the phylogenetic relationships of the lactobacilli were fully established, with

emphasis on the species of economic interest used by the food and feed industry.

There are many reports on the use of this technique, and mostly they have served the proper description of new species or contributed to the reduction of previously heterogeneous taxa and therefore proved to be indispensable for species determination within the genus *Lactobacillus.*

(i) *L. delbrueckii* group. On the basis of DNA-DNA hybridizations, two subgroups have been delineated. Subgroup 1 includes *L. delbrueckii*, with the three subspecies *delbrueckii*, *bulgaricus* (previously "*L. bulgaricus*"), and *lactis* (previously "*L. lactis*" and "*L. leichmannii*"), which share over 80% DNA similarity (344).

Subgroup 2 is represented by *L. acidophilus* sensu lato, which was shown to be composed of at least six species: *L. acidophilus* sensu stricto (133), *L. gasseri* (179), *L. crispatus* (31, 213), *L. amylovorus* (222), *L. gallinarum* (105), and *L. johnsonii* (105). This species classification is a result of several DNA-DNA hybridization studies (31, 105, 157, 178). Moreover, within subgroup 2, a DNA-DNA similarity of 13 to 44% was found between *L. helveticus* and *L. acidophilus* (157). This relatedness was confirmed by (i) a comparable base composition of the DNA, (ii) biochemical resemblance, and (iii) 16S rRNA sequence analyses (41). The synonymy between the former species "*L. jugurti*" and *L. helveticus* was already established (62, 282).

(ii) *L. casei-Pediococcus* group. Numerous DNA-DNA hybridization studies revealed discrepancies between species level identification by means of phenotypic tests and genotypic approaches. Only a few examples are given below.

When comparing the five subspecies of *L. casei* (230) by DNA-DNA hybridization (40), the majority of the subspecies *casei* strains, together with members of the subspecies *alactosus*, *pseudoplantarum*, and *tolerans*, shared high levels of DNA relatedness but were quite distinct (10 to 20% DNA similarity) from the type strain, *L. casei* subsp. *casei*. Therefore, a new species, *L. paracasei*, was proposed for all these organisms. Strains previously assigned to *L. casei* subsp. *tolerans* were phenotypically as well as genotypically sufficiently distinct to be maintained as a separate subspecies of *L. paracasei* (*L. paracasei* subsp. *tolerans*). *L. casei* subsp. *rhamnosus*, which formed a genotypically homogeneous group not related to the other members of *L. paracasei*, was elevated to the species rank as *L. rhamnosus*. The name *L. casei* (132) was restricted to the type strain only.

DNA-DNA hybridization studies performed by Dellaglio et al. (63) showed that *L. plantarum* was quite heterogeneous, since 8 strains (of the 28 strains investigated) displayed only 46 to 65% DNA relatedness to the type strain. These strains were shown to be highly related to *L. pentosus* (63), a species which was not included in the *Approved Lists of Bacterial Names* (284). In 1987, the latter species name was revived by Zanoni et al. (373). In 1991, Collins et al. (41) showed that the two species shared more than 99% 16S rRNA sequence similarity.

Vescovo et al. (339) found that 13 of 24 presumed *L. brevis* strains belonged to the species *L. hilgardii*, *L. kefir*, *L. confusus*, or *L. collinoides*, which indicates that the identification of *L. brevis*-like strains by carbohydrate fermentation reactions or additional simple phenotypic tests is insufficient. Farrow and Collins (94) investigated the genetic interrelationships of some strains from the human oral cavity and saliva, earlier assigned to *L. brevis* (54, 137, 139), and on the basis of nucleic acid and biochemical data, the strains were assigned to a new species, *L. oris.*

DNA base ratio. As mentioned above, the DNA base composition of lactic acid bacteria is less than 50 mol% G+C. This is a characteristic of the so-called *Clostridium* branch of the gram-positive bacteria, in contrast to the gram-positive bacteria of the *Actinomycetes* branch, which have a G+C content higher than 50 mol%. *L. pontis*, however, has a DNA base composition of 53 to 54 mol% G+C (341) and is phylogenetically very closely related to *L. reuteri*, with a G+C content of 40 to 43 mol% (162).

rRNA-targeted oligonucleotide probes. As an increasing number of DNA or rRNA nucleotide sequences become available, the comparison of homologous sequences can yield oligonucleotide stretches which are specific for the taxa being compared. These oligonucleotides can be labeled and used as probes in hybridization experiments with unknown isolates. By using PCR techniques, the target sequences can be amplified and the detection level can be enhanced. The use of probes, especially in food microbiology, has been reviewed by Schleifer (272). The value of this technique is demonstrated by the design of species-specific 23S rRNA targeted oligonucleotide probes useful for the identification of authentic *L. acidophilus*, *L. johnsonii*, *L. gasseri* (253), *L. delbrueckii*, *L. paracasei*, and *L. helveticus* and group-specific probes for *L. casei* and *L. rhamnosus* and for *L. casei*, *L. paracasei*, and *L. rhamnosus* (144). Oligonucleotide probes at the subspecies or genus level were also developed, e.g., to identify the genus *Lactococcus* (269) or the subspecies *Lactococcus lactis* subsp. *cremoris* (269), and *Lactococcus lactis* subsp. *lactis* (166).

Whole-cell protein analysis. The comparison of whole-cell protein patterns obtained by highly standardized SDS-PAGE has proven to be extremely reliable at the species and subspecies levels (67, 73, 144, 245, 250, 253, 254, 305, 327). It was possible to solve specific identification problems for, e.g., lactococci (67, 89, 154, 251), vagococci (250), *L. kefir* and *L. reuteri* (81), the species of the *L. acidophilus* group (253), and *Leuconostoc* strains (9, 82). The use of SDS-PAGE for the identification of a wide variety of lactic acid bacteria is hampered by the fact that it yields only discriminative information at or below the species level, requiring a certain degree of preidentification. This problem has been overcome by the creation of a database of digitized and normalized protein patterns for all known species of lactic acid bacteria (254). Identification of lactic acid bacteria is then reduced to the preparation of a protein pattern and a subsequent comparison with the patterns available in the database (245, 305, 327). The preparation of similarity dendrograms also yields information important for the classification of lactic acid bacteria (144, 253). The technique has proven to be extremely useful for grouping of large numbers of strains (67, 305). After numerical comparison, a limited number of representative strains can be selected from every (sub)cluster for further analysis by genotypic or phenotypic methods.

Lactate dehydrogenase. Lactate dehydrogenase (LDH) has been an important characteristic in the classification of lactic acid bacteria and has been studied in many ways.

The electrophoretic mobility of LDH in starch gels (106) or in polyacrylamide gels (143) was used mostly for the discrimination of phenotypically very similar species (e.g., *L. acidophilus*, *L. crispatus*, *L. gallinarum*, *L. gasseri*, and *L. johnsonii* [105]). A compilation of the relative migration distances of LDH of various species has been given by Kandler and Weiss (162) and more recently by Fujisawa et al. (105). NAD-dependent D-LDH profiles have also been used for the differentiation of *Leuconostoc* species (80). The following example shows, however, that care should be taken with the results of electrophoretic mobility tests of LDH. *L. jensenii* is indistinguishable from *L. delbrueckii* by simple physiological tests. Only a slight difference in the migration distance in starch gel electrophore-

sis of the D-LDH of the two species has been reported by using starch gel electrophoresis (106). This difference could not be demonstrated by polyacrylamide disk gel electrophoresis (162), and therefore the authors advised the use of the large difference in the moles percent G+C of the DNA (35 to 37 mol% for *L. jensenii* versus 49 to 51 mol% for *L. delbrueckii*) to differentiate the species.

Quantitative immunological techniques have been used with LDH as an evolutionary marker besides numerous other enzymes like fructose-1,6-bisphosphate aldolase (188, 190, 191), malic enzymes (192, 193), and glyceraldehyde-3-phosphate dehydrogenase (189). The data were used to create dendrograms and (three-dimensional) phylogenetic maps. On the basis of results with both D- and L-LDH, *L. acidophilus* sensu lato was divided into immunological groups I, II, and III (107), confirming the genotypic heterogeneity of this species discussed above.

Purification and characterization of the D-LDH from *L. delbrueckii* subsp. *bulgaricus* showed considerable sequence differences from representatives of the relatively well-characterized NAD-dependent L-LDHs (183).

Cell wall components. The phylogenetic distribution of different murein types in lactobacilli was comprehensively summarized by Hammes and Vogel (126). Homofermentative as well as facultatively heterofermentative lactobacilli from the *L. delbrueckii* group (groups Aa and Ba, respectively) all have the Lys–D-Asp type. The obligately heterofermentative organisms from the *Leuconostoc* group (group Cc) all have the Lys (monoamino, monocarbonyl) amino acid type (Lys–L-Ser–L-Ala_2 or Lys–L-Ala_2 [64]). Representative species of the *L. casei-Pediococcus* group (homofermentative [Ab], facultatively heterofermentative [Bb], and obligately heterofermentative [Cb]) have either the Lys–D-Asp type or the diaminopimelic-direct type. Only a few interesting exceptions occur: in *L. fermentum* and *L. vaginalis*, lysine is replaced by the chemically similar ornithine (Orn–D-Asp type); the Lys-Ala type is found in *L. sanfrancisco*; and the Orn–D-Asp type is found in *L. pontis.*

Conclusion. The phylogenetic structure of the lactic acid bacteria, as deduced from rRNA sequencing, is at present clear but is different from the physiological and chemotaxonomic classification schemes used for almost a century. Most of the taxonomic revisions were proposed during the last decade and are increasingly dependent on rRNA sequence information. However, because of the widespread use of relatively fast commercialized systems for testing phenotypic characteristics and the large number of taxa, the traditional phenotypic analysis remains important. The subdivision of lactobacilli (and other lactic acid bacteria) into three main fermentation categories (homofermenters, facultative heterofermenters, and obligate heterofermenters) is still used to identify strains. The discrepancies between phenotypic data present in traditional microbiology textbooks and phylogenetic data that have recently become available have not sufficiently been translated into new strategies for future work. Within the group of lactic acid bacteria, the use of phylogenetic methods has provided a highly reliable basis for the reevaluation of the phenotypic classification schemes; a result is the effective phenotypic description of new genera like *Oenococcus* and *Weissella*, which both form well-delineated phylogenetic and phenotypic entities.

For the study of the relationships at the species level, given a general identification by classical methods, several techniques are available. (i) The first is DNA-DNA hybridizations, which were useful and helped to select reliable reference strains for the rRNA sequence analysis. They are essential to study inter- and intraspecific relationships which are not revealed by rRNA sequence analysis. (ii) SDS-PAGE of whole-cell proteins has proven to be extremely reliable in revealing relationships at the species and subspecies levels. Since it is fast and cheap, this technique is well suited for identification of even large numbers of strains. Provided that a database covering all known species is available (254), the presence of a new taxon can be easily established. To estimate observed similarity in quantitative genotypic terms, however, DNA-DNA hybridization and rRNA sequence analysis are required. (iii) rRNA probe hybridization is a promising tool, especially if it is coupled to a PCR amplification step. The limitations at present are twofold. Because of the conserved nature of the rRNA molecule, cross-reactions between closely related species occur. In addition, the variation in hybridization procedures is large, which renders this approach for the identification of large numbers of strains quite complex.

For the identification of unknown isolates, it is necessarily a combination of these techniques that may actually prove to be the most rewarding with respect to accuracy and expenditure of time and labor.

Polyphasic Taxonomy of the Family *Comamonadaceae*

The family *Comamonadaceae* contains saprophytic and phytopathogenic bacteria with diverse physiological profiles (chemoorganotrophic or chemolithotrophic at the expense of H_2 or CO oxidation) (353, 354). They have been isolated from a wide variety of ecological niches such as soil, water, natural and industrial environments (353, 354), clinical samples, and infected plant material (240, 354, 356) and have an oxidative metabolism in which O_2 is used as the terminal electron acceptor, while some species can also use nitrates. Because of their physiological flexibility, they may play an important role in the biodegradation of xenobiotics (24) and recalcitrant components such as oil-derived wastes (1) and pollutants such as nitrate (311).

This family is currently composed of the genera *Comamonas*, *Acidovorax*, *Variovorax*, *Hydrogenophaga*, and *Xylophilus* and several misclassified *Aquaspirillum* species. Its core originated from the so-called *Pseudomonas* rRNA group III, one of the five distinct rRNA homology groups found in *Pseudomonas* species by the initial rRNA studies of Palleroni et al. (242). The phylogenetic distance between each of these rRNA groups was later (68) correctly interpreted by the inclusion of representatives of a wide variety of members of the *Proteobacteria* (291) in the DNA-rRNA hybridization experiments. Further investigation by comparative rRNA similarity studies (70, 72) illustrated the phylogenetic diversity of named *Pseudomonas* members compiled in addenda I through IV of Doudoroff and Palleroni (83). An important number of these species with uncertain taxonomic affiliation could be assigned to one of the five rRNA groups mentioned above, while others were scattered over the entire class of *Proteobacteria* (70, 72), belonging to existing or new taxa, which implemented a complete reclassification of the genus *Pseudomonas* (240), which is now restricted to rRNA group I organisms because the type species *Pseudomonas aeruginosa* belongs to it. This nomenclatural rearrangement of the genus *Pseudomonas* entailed the creation of several new genera encompassing either a complete rRNA group, e.g., *Brevundimonas* (278) or parts of rRNA groups such as *Burkholderia* (111, 367), *Stenotrophomonas* (241), *Hydrogenophaga* (351), *Acidovorax* (356, 360), etc. An overview of the updated taxonomic position of the pseudomonads will be presented elsewhere (69).

The phylogenetically related group of the *Comamonadaceae*

is also known as the acidovorans rRNA complex. As mentioned above, it originally consisted of the misclassified pseudomonads from *Pseudomonas* rRNA group III (68, 242): *P. acidovorans*, *P. testosteroni*, *P. delafieldii*, *P. flava*, and *P. palleronii*. Later, it was shown that this rRNA complex encompasses a variety of physiologically and morphologically diverse organisms, including rods and helical cells which are polarly, lophotrichously, or peritrichously flagellated (71, 72, 351, 353–361). The grouping of these apparently unrelated phenotypes into a single family was based on rRNA similarity data only. Apart from the misclassified *Pseudomonas* species, other members of the *Comamonadaceae* were wrongly assigned to genera (e.g., *Xanthomonas*, *Aquaspirillum*, and *Alcaligenes*) of which the type species belong to other rRNA branches of the *Proteobacteria*. It was also shown that *Comamonas terrigena* belonged to a separate rRNA subbranch and thus deserved a separate generic rank. As a consequence, the genus *Comamonas* and the species *Comamonas terrigena* were revived (71). *P. acidovorans* and *P. testosteroni*, belonging to the same rRNA subbranch as *Comamonas* species, were transferred to *Comamonas* as *C. acidovorans* and *C. testosteroni*, respectively (300).

rRNA similarities. Indirectly measured rRNA similarities (58) were used to unravel the phylogenetic structure of the *Comamonadaceae*. By using 15 different rRNA probes from well-selected strains, 14 distinct rRNA subbranches were delineated, each with a $\Delta T_{m(e)}$ range of 4 to 5°C (353); each theoretically deserved a separate generic status. Together, the 14 distinct rRNA subbranches constitute a separate lineage with $\Delta T_{m(e)}$ values that are similar or slightly lower than those observed in several other bacterial families including the *Neisseriaceae* [$\Delta T_{m(e)}$ range, 7.6°C (267)], the *Alcaligenaceae* [$\Delta T_{m(e)}$ range, 6°C (61)], the *Acetobacteraceae* [$\Delta T_{m(e)}$ range, 5°C (59)], and the *Pasteurellaceae* [$\Delta T_{m(e)}$ range, 8°C (60)]. Consequently, this separate lineage was proposed as a new bacterial family, the *Comamonadaceae*, and the internal similarities of the whole rRNA complex (of which the members of the *Comamonadaceae* constitute only one of the rRNA lineages) are shown in reference 353. The assignment at the family level was arbitrarily restricted to organisms with a $T_{m(e)}$ of at least 75°C when hybridized with the rRNA from the type strain of *C. acidovorans*. The grouping obtained by DNA-rRNA hybridizations was in agreement with 16S rRNA cataloging, since several members of the *Comamonadaceae* form a relatively tight cluster (364). Among the various members of the *Comamonadaceae*, only the 16S rRNA sequence of *C. testosteroni* is available. It constitutes a separate lineage in the beta subclass, with *Rhodocyclus gelatinosus* as its closest neighbor. Recent 16S rDNA sequences of more members of this family (211) are in general agreement with the DNA-rRNA hybridization results.

The internal taxonomy of the *Comamonadaceae* at the generic and species levels was clarified by investigating more than 150 strains belonging to the 14 rRNA subbranches with various genotypic and phenotypic methods. Table 4 gives an overview of the taxa belonging to the *Comamonadaceae* and illustrates the discriminatory power of the different methods used. The results of each technique were analyzed numerically, and the composition of the groups obtained by all methods was evaluated. Nomenclatural changes at the genus and species levels were proposed only when there was a consensus between the data obtained by the different techniques and when phenotypic data allowed clear-cut differentiation of the taxa to be proposed. Five genera covering nine rRNA subbranches were proposed: *Comamonas* encompasses four rRNA subbranches, *Acidovorax* encompasses two, while *Variovorax*, *Hydrogenophaga*, and *Xylophilus* each represent a single subbranch. Four of the remaining rRNA subbranches each contain a misclassified *Aquaspirillum* species: *A. delicatum*, *A. gracile*, *A. metamorphum*, and *A. anulus*. *A. sinuosum* and *A. giesbergeri* are closely related (150, 171, 252, 256) and belong to the last rRNA subbranch. *A. metamorphum* is equally far removed from the other rRNA subbranches and constitutes the core of an additional rRNA subbranch.

Because features such as *meta* cleavage of protocatechuate, growth at 41°C, absence of denitrification, and typical regulation of aromatic amino acid biosynthesis (see below) were not studied for all species, the phenotypic description of the family *Comamonadaceae*, which was created merely in terms of DNA-rRNA hybridization data, is still incomplete (353).

Recently, a new genus, *Ideonella* (199), has been proposed for new isolates that are capable of growing anaerobically with chlorate as an electron acceptor. They belong in the beta subclass, with 89 to 90% rRNA sequence similarity with *C. terrigena*, *Alcaligenes eutrophus*, and *Burkholderia cepacia*. Additional phylogenetic data on the other members of the *Comamonadaceae* are needed for comparison to reveal the relationship of *Ideonella* and the *Comamonadaceae*.

DNA-DNA hybridizations. DNA relatedness was used as the genotypic parameter to delineate species, but we have reservations about the absolute application of the 70% rule to species recognition (343), as discussed elsewhere. By using the initial spectrophotometric renaturation rates method (57), species were defined as groups of strains sharing at least 40% of DNA binding (356).

The selection of strains is important, and only a few representative strains of the different groups have been used in DNA-DNA hybridization experiments (351, 356, 357, 360, 361). The strains were selected as consensus representatives of clusters obtained by different techniques (SDS-PAGE of cellular proteins, cellular fatty acid analysis, and phenotypic and chemotaxonomic methods) used to screen large numbers of strains. When the groupings obtained by the different techniques did not match, strain selection was enlarged to represent all nonmatching clusters.

Nomenclatural changes were proposed only when consistent phenotypic evidence was available to describe and differentiate between the taxa. In some cases (e.g., *C. terrigena*), DNA-rRNA and DNA-DNA hybridization data could not be confirmed by phenotypic differences. Consequently, a single species was proposed for the *C. terrigena* phenon (361) containing three genomic groups. *A. aquaticum* belongs to one of them and lost its nomenclatural status. In the genus *Acidovorax*, the species delineation was hindered by the fact that the former *P. delafieldii* and *P. facilis* were shown to be phenotypically and chemotaxonomically more similar to each other than to *A. temperans*, a new species constituting a separate rRNA subbranch with the former two species. However, DNA-DNA hybridizations revealed separate DNA groups (DNA binding below 35%) for both *Pseudomonas* species, which were consequently assigned to two different *Acidovorax* species (351). Within *A. delafieldii*, at least two subgroups, showing 40 to 50% of DNA relatedness, were delineated. New *Acidovorax* isolates (110) showed 40% DNA binding with *A. facilis* as well as with *A. delafieldii*, suggesting that both species constitute a kind of genotypic continuum. Identification at the species level became difficult, if not impossible. This means that *A. facilis* and *A. delafieldii* must either be united into one species or can be considered to constitute a number of taxa deserving separate species status if they can be discriminated phenotypically. In view of the high phenotypic similarity between the species, a fusion of the species seems the more appropriate response.

TABLE 4. Polyphasic taxonomy in the family *Comamonadaceae*[a] including results and taxonomic resolution of the techniques used

Genus, species, and subspecies	rRNA branch[b]	Methods for intrageneric species and/or infraspecific differentiation[c]
Acidovorax		
delafieldii, *facilis*, *temperans*	8	ARDRA, SDS-PAGE,[d] DNA-DNA, FAME, and auxanography
avenae subspp. *avenae*, *cattleyae*, and *citrulli*) and *konjaci*	14	ARDRA, SDS-PAGE,[d] DNA-DNA,[d] and auxanography
Comamonas		
acidovorans	1	ARDRA, SDS-PAGE, DNA-DNA, immunotyping, and auxanography
testosteroni	2	ARDRA, SDS-PAGE, DNA-DNA, immunotyping, and auxanography
terrigena DNA hybridization group 1	3	rRNA,[d] ARDRA,[d] SDS-PAGE,[d] DNA-DNA,[d] immunotyping,[d] and auxanography
terrigena DNA hybridization group 2	4	rRNA,[d] ARDRA,[d] SDS-PAGE,[d] DNA-DNA,[d] immunotyping,[d] and auxanography
terrigena DNA hybridization group 3	sep[e]	rRNA,[d] ARDRA,[d] SDS-PAGE,[d] DNA-DNA,[d] immunotyping,[d] and auxanography
Hydrogenophaga		
flava, *pseudoflava*	6b	ARDRA, SDS-PAGE,[d] and auxanography
palleronii	6a	ARDRA, SDS-PAGE,[d] DNA-DNA, auxanography, and FAME
taeniospiralis	6	ARDRA, SDS-PAGE,[d] DNA-DNA, and auxanography
Variovorax		
paradoxus	7	
Xylophilus		
ampelinus	5	
Aquaspirillum		
anulus	9	Classical phenotyping[f]
giesbergeri, *sinuosum*	12	Classical phenotyping[f]
metamorphum	11	Classical phenotyping[f]
delicatum	10	Classical phenotyping[f]
gracile	13	Classical phenotyping[f]
psychrophylum	sep[e]	Classical phenotyping[f]

[a] The results are from references 239, 268, 296, 300, 351, and 353–361.
[b] Genera or species belonging to different rRNA branches can be differentiated by their rRNA. The 14 rRNA branches of the *Comamonadaceae* have been numbered in Fig. 1 in reference 353 from top to bottom.
[c] ARDRA, amplified rDNA restriction analysis; SDS-PAGE, analysis of electrophoretic protein patterns; FAME, analysis of fatty acid methyl ester profiles; DNA-DNA, DNA-DNA hybridization.
[d] Allows infraspecific differentiation.
[e] At the base of the 14 rRNA branches in the *Comamonadaceae.*
[f] According to Krieg (171).

Amplified rDNA restriction analysis. The value of amplified rDNA restriction analysis for identification of the phylogenetically and phenotypically delineated genera and species within the *Comamonadaceae* has been investigated (331). Restriction analysis of the 16S rDNA, the 16S to 23S rDNA spacer region, and part of the 23S rDNA with *Hin*fI and *Cfo*I resulted in consistent species-specific patterns (331), suggesting that identification at the species level is possible. Within *C. terrigena*, the three genotypic groups could be differentiated by the combined use of *Hin*fI and *Nci*I patterns.

DNA base ratio. The mean percent G+C content of representative members of all genera and species of the *Comamonadaceae* was determined. The range within this family is from 57 to 70%. The lowest values (57 to 58%) were found for some of the misclassified *Aquaspirillum* species (361). Within the different genera, the moles percent G+C range varies from 1% in *Xylophilus* (359) to 9% in *Comamonas* (361).

Whole-cell protein analysis. In several rRNA subbranches, the numerical interpretation of the profiles of SDS-PAGE of whole-cell proteins allowed us to group large numbers of genotypically related strains. Fairly stable groups were usually obtained, which corroborated DNA hybridization groups (256, 351, 355, 356). In some genera, e.g., *Xylophilus* (359) and *Comamonas* (361), individual strains showed aberrant profiles because of the presence of one or more heavy protein bands that affected the numerical interpretations.

Numerical analysis of phenotypic features. Phenotypic analysis based on classical physiological and morphological characteristics and a wide variety of carbon assimilation tests performed in miniaturized commercially available galleries (bio Mérieux, Montalieu-Vercieu, France) allowed us to cluster the strains by computer-assisted numerical interpretations into different phena (351, 356–358, 360, 361). When the phena corroborated the clustering found by other techniques, the groups involved were classified and the phenotypic results were used to discriminate them at the appropriate taxonomic level. If the phena did not support the clustering obtained by other screening techniques and by DNA-DNA hybridization, the phenotypic grouping was considered decisive for taxonomic conclusions.

Immunotyping. The immunotyping technique (319) as designed by Falsen (Culture Collection of the University of Göteborg, Göteborg, Sweden) was extensively used to unravel the internal structure of *Comamonas* and *Hydrogenophaga* species.

For *Hydrogenophaga* species (351), immunotyping confirmed the close relationships of the yellow hydrogen oxidizers originally and erroneously classified as *P. flava*, *P. pseudoflava*, *P. carboxydoflava*, *P. taeniospiralis*, and *P. palleronii. P. pseudoflava* and *P. carboxydoflava* even showed an identical precipitation pattern. Furthermore, *A. delafieldii* and *A. facilis* turned out to be the only members of the *Comamonadaceae* to react, although to a minor degree, with the antiserum against *Hy-*

drogenophaga pseudoflava. This suggests again a closer relatedness between *A. facilis* and *A. delafieldii*, which was also found by phenotypic and chemotaxonomic methods but which could not be confirmed by DNA-DNA hybridizations (356).

Cellular fatty acid analysis. Cellular fatty acid analysis was applied only to a limited number of strains of certain species, and different methods have been used (238, 239, 268, 296, 300, 356). Therefore, general conclusions about the suitability of this method to differentiate all members of the *Comamonadaceae* cannot be drawn (Table 4). The major fatty acids are palmitoleic acid (16:1 *cis* 9), palmitic acid (16:0) and *cis*-vaccenic acid (18:1 *cis* 11) (238, 239, 268, 296, 300, 353). The absence of 3-hydroxypalmitic acid (16:0 3-OH) has a limited value to discriminate the members of the *Comamonadaceae* from other related groups (353). Two fatty acid groups were discriminated by Stead (296) and by Sakane and Yokota (268): one without 2-hydroxy acids (*C. acidovorans*, the plant-pathogenic members, and the *Aquaspirillum* species) and one (*C. testosteroni*) containing small amounts of several 2-hydroxy acids. Tamaoka et al. (300) differentiated *C. acidovorans* and *C. testosteroni* by measuring the levels of myristic acid (14:0). The comparison of fatty acid profiles was intensively used in the genera *Hydrogenophaga* and *Acidovorax* (351, 356). Numerical analysis revealed that most species investigated form rather homogeneous clusters. However, both interspecies and intergeneric distances, as measured by this technique, appeared to be uncorrelated with the genetic relatedness observed in *Hydrogenophaga* and *Acidovorax* species (351, 356).

Polyamine patterning. Since polyamine patterning was not used on a representative selection of all species in the family *Comamonadaceae*, its ability to discriminate the species and genus levels is unclear. All members of the β-subclass of the *Proteobacteria* are characterized by the presence of 2-hydroxyputrescine (24, 25, 125). In the genus *Comamonas*, the polyamine profile was rather simple and contained only hydroxyputrescine and putrescine as major components, together with a small amount of spermidine.

Other features. Low-molecular-weight RNA profiles allow us to differentiate *Comamonas* and *Hydrogenophaga* species from the rRNA group I pseudomonads (148). A ubiquinone with eight isoprenoid units on the side chain was found to be the major respiratory quinone component in all members of the *Comamonadaceae* tested (39, 238, 239, 268). Enzymes which participate in the biosynthesis of aromatic amino acids exhibit a diverse assemblage of qualitative "character states," whose distribution can be traced in a phylogenetic progression. The concordance of the phylogenetic tree based on 16S rRNA and the clustering observed for character states of aromatic amino acids biosynthesis has been documented (26, 27, 350). Recent studies have shown that *C. testosteroni*, *C. acidovorans*, *Hydrogenophaga facilis*, and *P. saccharophila* constitute a cohesive group sharing the same distinct character states for aromatic biosynthesis (297). The electrophoretic pattern of different enzymes was used to characterize *C. terrigena* and *C. testosteroni* (300).

Conclusion. The physiologically and morphologically diverse group of the *Comamonadaceae* constitutes one phylogenetic lineage as measured by rRNA similarities containing 14 rRNA subbranches. Each subbranch is genotypically sufficiently distinct to represent a separate genus. A combination and evaluation of the data obtained by the stepwise use of techniques with complementary discriminative power were applied to further unravel the inter- and intrageneric similarities within this family.

A number of rRNA subbranches have been united in one genus, e.g., *Comamonas* and *Acidovorax*, while for others (*Xylophilus*, *Hydrogenophaga*, and *Variovorax*), a single genus corresponds to one rRNA subbranch only. Also, on the species level, new taxa were not created when phenotypic differentiation was not possible (e.g., *C. terrigena*). Despite their separate genotypic positions, we avoid proposing nomenclatural changes for the different misclassified *Aquaspirillum* species, because each branch is composed of only a few strains and because not enough phenotypic data are available to clearly differentiate these branches from all the other aquaspirilla (256).

Strategies for polyphasic identification of unknown isolates within the *Comamonadaceae* can be deduced from Table 4. For family and genus level allocation, it is clear that rRNA information is needed. For differentiation between most species, DNA-DNA hybridizations are recommended, although different fingerprinting techniques (Table 4) often allow reliable species identification.

EVOLUTION OF POLYPHASIC TAXONOMY AND PERSPECTIVES

The problem of classifying bacteria into orderly arranged taxa was initiated in the previous century (34, 35). The odyssey of bacterial classification is characterized by several milestones which led toward our present insights and ideas. Minimal numbers of phenotypic characters were replaced by large numbers of phenotypic and genotypic data which rendered classification systems more stable and which are now embedded in polyphasic classification systems. Taxonomic studies have often revealed that classifying bacteria by a single approach conflicted with results of polyphasic analyses. These monophasic approaches include the ancient "form" classification and pathovar systems and also rRNA sequence analyses, as discussed below. A classification which considers all information on all known aspects of a particular organism covers essential parts of its genome. It can be argued that this is the underlying reason for its superior stability.

Every classification should be an attempt to arrange the natural diversity among strains into a hierarchical system. Different taxonomic levels are discriminated to score the extent of divergence. Variability among organisms is expressed in numerous different molecules, which explains why monophasic classification systems are bound to fail. Any character which reveals part of this variability is therefore useful and can be considered. Experience showed, however, that some of these characters have superior value and are relevant parameters for particular taxonomic ranks. Some of these characters are discussed in detail below, because their application in polyphasic approaches is not always straightforward.

DNA Hybridization Studies

At present, the bacterial species concept is generally accepted among taxonomists, and DNA hybridization is acknowledged as the reference method to establish relationships within and between species. Wayne et al. (343) defined a species as an entity which included ". . .strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m. . ." Both values were to be considered, and phenotypic characteristics had to agree with this definition. This species definition was based on a large amount of experience combining both DNA hybridization data and other features.

Although proclaimed as the "gold standard" to delineate bacterial species (343), the technique of DNA-DNA hybridization did not gain large popularity, and complete triangles of hybridization values between related species are extremely rare. A complete triangle based on 790 hybridization values for

183 strains was given for *Xanthomonas* species (332). DNA-binding values for strains belonging in the same DNA hybridization group were never below 60%. The average levels of DNA binding between strains of different DNA hybridization groups were typically less than 40%, although values of 50% also occurred. Analysis of such observations suggests an uneven distribution in taxonomic space, with clusters of highly related strains that have more than 80% DNA-binding values (332).

DNA hybridization techniques present severe disadvantages because they depend on physicochemical parameters, are not cumulative, are cumbersome, and require great quantities of DNA (287). As long as DNA-DNA hybridization experiments form the cornerstone of the bacterial species definition, there will be a need for a new, more rapid, miniaturized, automated, and standardized method. DNA hybridization data obtained in various laboratories do not always correlate well, partly because different methods give different results (121) but also because hybridization data are not always obtained under strictly comparable conditions (optimal, stringent, or suboptimal). Examples of such lack of correlation are found when comparing results of DNA hybridization studies among campylobacters. One of the rRNA clusters within the genus *Campylobacter* comprises *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* (243, 321) and has been the subject of DNA-DNA hybridization studies. Using the hydroxyapatite method, Ursing et al. (308) reported low but significant DNA-binding values between *C. upsaliensis* and *C. jejuni* (36 to 43%), *C. upsaliensis* and *C. coli* (23 to 44%), *C. lari* and *C. jejuni* (29 to 40%), and *C. lari* and *C. coli* (26%). However, Roop et al. (264, 265), using the S1 nuclease procedure, detected significant DNA-DNA hybridization values only between *C. jejuni* and *C. coli* (25 to 49%) and detected values up to 20% between, e.g., *C. lari* and *C. jejuni*, but similar values were also found between *C. lari* and several other *Campylobacter* species from other rRNA clusters such as *C. fetus* and *C. sputorum*. Comparison of these values with the percent difference in 16S rRNA sequence homology confirms the importance of the DNA-DNA hybridization technique and the conditions used. *C. upsaliensis* differs in 5% of its 16S rRNA sequence from *C. coli* (294), whereas about 40% DNA-DNA binding is detected by the hydroxyapatite method (270). On the other hand, *C. coli* and *C. jejuni* differ only in 1.5% of their 16S rRNA sequence (294), and, again, about 40% DNA-DNA binding is detected by the S1 nuclease procedure (264).

Obviously, quantitative comparisons of DNA hybridization values generated by different techniques should be handled with extreme caution. As stated above, the conditions for hybridizations are extremely important and only results obtained under comparable conditions can be evaluated objectively. It might therefore be safer for such comparisons to distinguish three categories of DNA-DNA relatedness, such as high DNA relatedness, which would denote relationships between strains of a single species; low but significant DNA relatedness, which would denote the range of significant hybridization values below the species border (the depth of this range depends primarily on the technique used); and, finally, nonsignificant DNA relatedness, which would indicate that the degree of DNA hybridization is too low to be measured by the method used. As discussed above for *A. delafieldii* and *A. facilis*, it is not easy to discriminate low but significant DNA binding from nonsignificant DNA binding, which can lead to confusion in the species delineation and hinder the identification.

Regardless of the hybridization method used, serious difficulties were encountered in applying the 70% DNA hybridization rule. It was recommended (343) that ". . .a distinct genospecies that cannot be differentiated from another genospecies on the basis of any known phenotypic property not be named until they can be differentiated by some phenotypic property. . ." Logically, it is practical to differentiate species on a routine basis only if they can be readily differentiated. Preferentially, a number of simple and straightforward tests (at least two [10]) should endorse species determination based on DNA hybridization values. Phenotypically similar but genotypically distinct groups of strains have been referred to as genomic species, genomic groups, genospecies, genomospecies, or genomovars (309). *Comamonas terrigena* is such an example, since it contains three clear-cut genotypic groups constituting one phenotype. The name "genomovar" may be misleading, because these taxa are given an infraspecific rank, whereas they are to be considered by definition to be species that cannot be reliably differentiated by phenotypic tests. Aside from the taxonomic categories species, genus, and family, a number of additional categories have been introduced, e.g., biotype, biovar, subspecies, and genomovar. They often created more problems than they resolved, functioning more as smoke screens to hide uncertainty, conceptual weakness, or lack of imagination. Although these are sometimes initially meant as temporary tools to handle unsolved problems, taxonomic relationships will not be clarified by merely introducing additional categories, terminologies, or both. The level of 70% binding and 5°C ΔT_m is very strict, and often phenotypic consistency would not be achieved if these recommendations were strictly applied. Ursing et al. (309) considered species to be groups of strains sharing 50 to 70% DNA reassociation and 5 to 7% difference in thermal stability between the homologous and heterologous duplexes, which clearly is a more realistic standard. Even with this less strict definition, many species of different phylogenetic lineages contain genomovars. Although the presence of genomovars is obviously too important to be ignored, it is just not practical to create additional names for groups which cannot be readily identified, and it was proposed to denote these genomovars by using numerals, not names (309).

In other cases, DNA hybridization values conflicted with data derived from other analyses. The genus *Bordetella* presents a striking example. *Bordetella* comprises an extensively studied group of gram-negative bacteria, closely related to the genus *Alcaligenes* (61) and comprising six species (322, 349): *Bordetella pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. avium*, *B. hinzii*, and *B. holmesii*. All these species are readily differentiated by means of classical phenotypic tests and whole-cell protein analysis, and most of them have clearly different whole-cell fatty acid components as well (314, 315, 322). Particularly *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are, in laboratory practice, very different organisms, which are easily differentiated by several morphological, biochemical, and chemotaxonomic characteristics. However, these three species share more than 80% DNA binding (167, 322, 349). If there were indeed a simple linear relationship between thermal stability and DNA mispairing as described by Bautz and Bautz (13) and Ullmann and McCarthy (306), a tremendous amount of information must be encoded in a limited part of the *Bordetella* genome. Cases like this were foreseen in the guidelines of Wayne et al. (343), which state that phenotypic characteristics can override the phylogenetic concept of species in a few exceptional situations.

rRNA Sequence Analysis

It is well documented that the deep phylogenetic relationships between living organisms can be deduced from sequence

comparisons of rRNA molecules and from some of their signature features (123, 226, 363). Comparisons of the 16S and 23S rRNA sequences revealed the phylogenetic framework of bacterial classification and became indispensable in polyphasic taxonomy. Analysis of partial sequences, although valuable, should preferentially not be used for taxonomic conclusions unless it has been shown that they give the same degree of similarity as that obtained with full sequences (287). The different phylogenetic positions of *Rhizobium galegae* obtained by partial and total 16S rDNA sequences (223, 352) support this. Total rDNA sequences are accumulating rapidly and are accessible via international databases (66, 226). The presence of universal bacterial sequences in the rRNA molecule allowed us to classify unculturable organisms and to perform phylogenetic identifications and in situ detection of individual cells without cultivation (3). More recently, rRNA sequence analysis was no longer used exclusively to determine relationships between genera, families, and other higher ranks but also for species delineation and increasing numbers of new species are described without DNA hybridization studies or equivalent techniques (see below).

Within this wave of enthusiasm, a number of dissonant voices were recorded. In 1992, Fox et al. (102) reported that 16S rRNA sequence identity may not be sufficient to guarantee species identity. Three phenotypically similar *Bacillus* strains exhibited more than 99.5% rRNA sequence similarity (rRNA sequences are considered identical if they differ in less than 5 to 15 positions [102]), whereas DNA hybridization experiments indicated that they belonged to two distinct species. It has been proposed to refer to such taxa with (virtually) identical rRNA sequences as rRNA species complexes or rRNA superspecies (102).

In 1994, Stackebrandt and Ludwig (289) discussed the importance of choosing outgroup reference organisms in phylogenetic studies. It is more than relevant to know that the branching order in phylogenetic trees depends not only on differences in base composition, tempo of evolution, unequal rates of evolution in different regions of the rRNA genes, and selection of sequence stretches analyzed but also on the number of organisms and the selection of reference organisms. Therefore, it is best to include a wide range of apparently related and apparently unrelated reference organisms. It is important not to restrict the selection to a large number of apparently related taxa, even if this seems to be common practice. It is recommended to determine the significance of the branching levels in a phylogenetic tree by means of bootstrap analysis (173).

Stackebrandt and Goebel (287) presented a taxonomic note on the place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Their review of the literature data revealed that, in general, organisms sharing more than 97% rRNA similarity may belong to a single species and that the resolution of 16S rRNA sequence analysis between closely related organisms is generally low. There is simply no threshold value of 16S rRNA homology for species recognition (287). However, organisms with less than 97% 16S rRNA sequence homology will not give a DNA reassociation of more than 60%, no matter which DNA-DNA hybridization method is used. Therefore, in fact, rRNA sequence analysis may replace DNA hybridization studies as part of the description of new species, provided that the rRNA similarity level is below 97% and that rRNA sequence data of all relevant taxa are available for comparison.

Clearly, one should be prudent with conclusions based on single-sequence analysis. In 1995, Clayton et al. (33) presented a detailed comparison of duplicate rRNA sequences present in the GenBank database (Release GenBank87; 15 February 1995), with remarkable results. Unexpectedly high levels of intraspecies variation (within and between strains) of 16S rRNA sequences were found. The variability was thought to represent interoperon variation within a single strain, strain-to-strain variation within a species, misidentification of strains, sequencing errors, or other laboratory errors (33). Analysis of the available data revealed interoperon differences from 0 to 5% and interstrain differences from 0 to 16%, which appears to undermine the application of rRNA sequences for phylogenetic and identification purposes and to question the ability of this method to identify unculturable organisms or to study microbial communities without the need for cultivation (3). However, in numerous reports, the conclusions based on rRNA sequence analysis confirmed results obtained by equivalent techniques such as DNA-rRNA hybridizations (in which several strains were usually included) or rRNA cataloging (cf. the examples of polyphasic taxonomy described above) or were substantiated by a polyphasic approach on a large number of strains. This indicates that although the scientific rationale of rRNA sequence analysis cannot be doubted, the problem of errors in some sequences present in the EMBL database should not be underestimated.

Confirmation of the validity of an rRNA sequence requires repetitive assays, but for a well-studied taxon it may also be achieved by the selection and evaluation of taxon-specific primers from that sequence, which would avoid the need for multiple-sequence analysis and has the advantage that it is feasible to examine many strains. Alternatively, several other macromolecules have been examined for their potential as microbiological clocks. Among others, the beta subunit of ATPase (194), elongation factor Tu (194), chaperonin (340), various ribosomal proteins (224), RNA polymerases (374), and tRNAs (147, 148) were shown to be valuable molecular chronometers in bacterial systematics. These alternative macromolecules should be universally distributed among bacteria, their genes should not transmit horizontally, and their molecular evolution rate should be comparable to or somewhat higher than that of 16S rDNA, which would render them more suitable for differentiation of closely related organisms. The closing remark of Clayton et al. (33) covers both past and future classification systems and is indeed certainly valid for bacterial systematics: ". . .Assessing within-taxon variability of the characters examined has long been standard procedure in animal and plant systematics and ecology, whether features studied are morphological, behavioral, or biochemical. It should come as no surprise that nucleic acid sequences require similar scrutiny, especially in prokaryotes, a paraphyletic group in which systematic designations are so often provisional."

Phenotypic Data

In taxonomic practice, phenotypic characterization became compromised and sometimes more of a burden than a useful taxonomic activity. Frequently, phenotypic data are compared with literature data, which were or might have been obtained by using other conditions or methods. However, identification of microbes in the day-to-day practice of the routine microbiology laboratory relies nearly exclusively on those differential phenotypic features. The report of the Ad Hoc Committee on the Reconciliation of Approaches to Bacterial Systematics stressed that any phylogenetically based taxonomic scheme must also show phenotypic consistency (343). In fact, this is the bottom line of polyphasic taxonomy. The depth in an rRNA dendrogram at which a given hierarchical line is to be drawn should remain flexible to achieve phenotypic consistency. In

practice, this is not always possible, as illustrated above for the lactobacilli. In the case of the above-mentioned examples of genera delineated by polyphasic approaches, the depth expressed as a percent difference in 16S rRNA sequences corresponded to about 4% in the genus *Xanthomonas*, 10% in *Campylobacter*, 8% in *Helicobacter*, 6% in *Arcobacter*, and 5% in *Bordetella*. The genera in the family *Comamonadaceae* show a phylogenetic heterogeneity corresponding to a $\Delta T_{m(e)}$ of their DNA-rRNA hybrids of between 2 and 5°C. Since only few 16S rRNA sequences are available in this group, a maximum of 5% sequence dissimilarity is estimated indirectly. Similarly, polyphasic approaches combining rRNA sequence data and phenotypic data entailed the delineation of the families *Campylobacteraceae* at 16% (294, 317), *Neisseriaceae* at 7% (75), *Cardiobacteriaceae* at 8.5% (76), *Pasteurellaceae* at 6% (77), and *Alcaligenaceae* at 5.5% (84). Within the *Comamonadaceae*, the percent difference in 16S rRNA sequences between the genera is indirectly estimated at around 5 to 6%.

The need for a continued phenotypic characterization at every taxonomic level cannot be denied, not only to delineate taxa and appreciate their phenotypic coherence but also to evaluate their physiological and ecological functions. A minimal phenotypic description is not only the identity card of a taxon but also a key to its biology. Although they are accepted as necessary, differential phenotypic characters are often hard to find with a reasonable amount of effort and time. For *Campylobacter* and its relatives, the phenotypic inertness of the bacteria has prevented the development of a solid phenotypic identification scheme, whereas for lactobacilli, the large number of species and the overall phenotypic similarity between some closely related species (e.g., the *L. acidophilus* complex) did prevent a reliable method of species determination until genotypic methods were used. The phenotypic description of bacterial families presents even more problems, as was illustrated above for the *Comamonadaceae*.

It is impossible to estimate the variability of the phenotype in the one strain-one species case or in the one strain-one species-one genus case, for which many recent examples exist (see, e.g., references 19, 22, 65, and 197). The question whether to validly name such strains has been the subject of many debates. There are different views, each with advantages and disadvantages. In a thorough taxonomic study, a single strain can unambiguously be shown to represent a new genus or a new species within an existing genus. The genotypic and phenotypic characteristics of this strain can be determined and compared with those of strains of its nearest neighbors. For many taxonomists, this is sufficient to validly name this organism. However, the characteristics of one isolate cannot represent the phenotypic variability of an entire species, and therefore the description of the new species, which is at that moment represented by a single strain, cannot be adequate. It can be argued that a classification based on results obtained with a single strain cannot be stable and may warrant future emendations of descriptions, nomenclatural corrections, or both. Clearly, these modifications jeopardize the credibility of taxonomists in the microbiology community. In this discussion, two additional points should be mentioned. The same could be argued for the description of a single species which represents a new genus. The variability of the genus-specific characteristics can also not be estimated if only a single species is available. However, it is perfectly possible that a genus comprises only a single species, and therefore, the two situations cannot be validly compared. Alternatively, it is often very simple to obtain, mala fide or not, many isolates which do not represent different strains (e.g., daily samples of a single source).

The discussion will probably linger, but at present, it can be recommended that new species containing a single strain or new genera containing a single species be validly named only if their genotype and phenotype have been thoroughly and adequately characterized.

In light of the increasing application of phenotypic fingerprinting systems, it should be mentioned that the application of phenotypic fingerprinting systems (e.g., Biolog) and their inclusion in official descriptions restrict the examination of the bacterial phenotype to a minimum, thus also restricting the knowledge of the phenotype. This problem is clearly illustrated by the newly described *Xanthomonas* species (332), which are based largely on Biolog data, and by the description of, e.g., *Acidovorax temperans*, which is based exclusively on auxanographic results obtained by an API system which is no longer commercially available in the same format. The limited consensus between results obtained by classical phenotypic growth tests and commercial systems is the origin of the decrease in the availability of reliable tests for the description and identification of new species, as, e.g., for *Burkholderia* species (111).

Whole-Cell Fatty Acid Analysis

Whole-cell fatty acid analysis is increasingly used, both in taxonomic studies and in identification analyses (345). The applications and restraints of the technique were extensively discussed and documented by Welch (345). In the framework of polyphasic taxonomy, cellular fatty acid analysis is often very useful as a rapid and fairly inexpensive screening method. The procedures for extraction, chromatographic separation, and data analysis are relatively simple and highly automated. The method therefore allows the comparison and clustering of large numbers of strains with minimal effort and yields descriptive information to characterize the organisms. It is important to determine the resolution level of this technique for every taxon under investigation. For some genera, whole-cell fatty acid analysis allows differentiation and identification of individual species, or even subspecies, while for others, different species have identical fatty acid profiles (345). Therefore, depending on the group of bacteria studied, similar fatty acid patterns may be shown by diverse taxa representing different taxonomic ranks. Importantly, strains must be grown under highly standardized conditions to obtain reproducible results. The commercial microbial identification system (Microbial ID Inc., Newark, Del.) gives strict guidelines and provides databases containing species-specific fatty acid patterns. However, in our experience, individual species within genera are often not differentiated, because when large numbers of strains are examined, some grow slowly, and it is often necessary to prolong the incubation period to generate sufficient cells for each strain under the same growth conditions. The differentiation of *Bordetella avium* and *B. hinzii* is an example of such a loss of resolution: cells of both species, grown for 24 h, were easily differentiated (46), whereas the same strains, grown for 48 h, were no longer distinguishable (322). Additional examples have been described by Welch (345). Prolongation of the incubation period cannot generally be considered a cause of reduced resolution, because in other groups such as *Aeromonas* and *Xanthomonas* species, the discriminatory power is considerably higher when strains are grown for 48 h than when they are grown for 24 h (149, 371).

Whole-Cell Protein Analysis

Numerous studies have documented the application of whole-cell protein electrophoresis to taxonomic studies (47, 164, 255, 335). The rationale for this method is (i) that strains, when cultivated under highly standardized conditions, will be

characterized by a protein composition which can be separated by electrophoretic techniques and visualized by staining procedures, and (ii) that a high similarity in whole-cell protein content is usually an indication of extensive DNA hybridization (47, 255). Therefore, an obvious advantage of this technique is that once the correlation between percent similarity in whole-cell protein composition and DNA relatedness for a particular group has been established, it can replace, in part, DNA hybridization experiments. It should be remembered, though, that in some groups, strains within a single species may have rather different whole-cell protein patterns and therefore that differences in whole-cell protein composition do not necessarily imply low levels of DNA hybridization (323).

Provided that highly standardized conditions are used throughout the procedures of cultivation and electrophoresis, computer-assisted numerical comparisons of protein patterns are feasible and databases can be created for identification purposes (164, 254, 318). This allows large numbers of strains to be compared and grouped in clusters of closely related strains (47, 255). For some bacteria, numerical analysis may be hindered by the presence of distorted protein profiles or hypervariable dense protein bands. In these cases, visual comparison is essential to interpret the similarity of protein patterns. For certain taxa (e.g., rhizobia), standardizing the growth period is not always possible, since closely related groups of strains can differ considerably in generation time (55).

For its application in polyphasic taxonomy, protein electrophoresis has an important drawback: in contrast to, e.g., whole-cell fatty acid analysis, it does not supply descriptive information, because in general, the identity of none of the protein bands is revealed.

DNA-Based Typing Methods

As described above, the introduction of molecular biological techniques into the microbiology laboratory yielded a large variety of DNA-based typing methods. Although most of these methods are particularly useful in determining infraspecific relationships, some of them have found broader taxonomic applications.

Amplified rDNA restriction analysis has been used to examine the taxonomic structure of a growing number of genera including *Streptococcus* (155), *Clostridium* (122), *Rhizobium* and *Bradyrhizobium* (174), *Brevibacterium* (29), and *Leptospira* (258) and the family *Comamonadaceae* (331). The available data reveal that this technique is useful primarily to identify strains at the species or infraspecific level. Numerical analysis of the patterns generated with several restriction enzymes provides supraspecific information as well. Obviously, the resolution of this method is determined primarily by the target of the PCR (e.g., if a spacer region is included, a higher discriminatory power will normally result).

The first results of the AFLP technique applied to *Xanthomonas* and *Aeromonas* species suggest its potential usefulness at the species and subspecies levels, as well as for the fine typing of individual strains (153).

PCR-based typing methods with random or repetitive elements as primers have been applied to strain characterization in a wide variety of bacteria (310). In several of these studies, species-specific DNA fragments were generated (e.g., for species belonging to the genera *Campylobacter*, *Capnocytophaga*, and *Naegleria* [108]). These specific DNA fragments may be useful as probes to rapidly screen and identify other isolates. Although these techniques are primarily applied to infraspecific strain comparisons, they may prove to be useful in classification as well.

Strategy in Polyphasic Taxonomy

From the examples and discussion given above, it should be obvious that there are no simple and straightforward guidelines for performing polyphasic taxonomic studies. The scope of the study is determined primarily by the problem itself. Classifying 100 strains without any preexisting knowledge of their possible identity and classifying a single unidentified *Bordetella* strain are totally different problems. Any chosen strategy may be influenced in the course of the study by the results obtained. Researchers who intend to perform polyphasic analyses should be equipped with techniques which allow them to determine the phylogenetic affiliation of an unknown isolate. Undoubtedly, at present, rRNA or rDNA sequence analysis is the best choice, although other macromolecules probably have a similar or even additional potential. Preferably, two different screening techniques should be available to compare and group large numbers of strains. The genotypic structure of such groups may be analyzed by DNA-based typing techniques, but ultimately, DNA-DNA hybridization studies of a limited number of well-chosen strains are required to delineate individual species. Finally, to provide the scientific community with useful information which is also easily accessible, the phenotype should be characterized.

Polyphasic Identification

Choosing a polyphasic approach to bacterial classification raises the question of identification of individual isolates. In this context, it should not be forgotten that most isolates in routine microbiology laboratories are identified without difficulty by using conventional phenotypic schemes. Databases of rRNA sequences and whole-cell fatty acid components and miniaturized batteries of phenotypic characteristics exist, allowing the identification of many isolates. However, the success of these databases depends on the exactness of the methods used and on how carefully the individual entries were delineated. In our view, a stable entry can only be the product of a polyphasic analysis which will reduce the amount of work for subsequent monophasic identification schemes. The classification of new or unusual isolates will, however, often require a polyphasic approach.

Unculturable Bacteria

Another remaining problem concerns the classification and nomenclature of unculturable bacteria which are only minimally characterized by morphological characteristics or by differences in a molecular sequence (220). In case the difference in 16S rRNA similarity with its closest neighbor is very large, it can be argued that the unculturable organism represents a novel taxon. However, this assumption does not take into account that a 16S rRNA or rDNA sequence is not available for all known bacteria for comparison. Alternatively, some bacteria were named just because they differed morphologically from the other bacteria present in the same habitat. As an example, unculturable bacteria morphologically different from *H. pylori* were found in the human stomach and named "*Gastrospirillum hominis*" (205), but later rRNA analysis showed that they belonged to the *Helicobacter* lineage, and so the species was renamed "*Helicobacter heilmanii*" (286). However, comparison of the 16S rRNA sequences of two of these organisms with those of other helicobacters revealed only 96.5% rRNA similarity between the two sequences and values of 96.6

and 98.8% toward *Helicobacter felis* (104), an organism which is extremely fastidious. In the same period, additional gastrospirilla were detected in the stomachs of a lemur and of pigs, and these were named "*Gastrospirillum lemur*" and "*Gastrospirillum suis*" (208), respectively. rRNA sequence comparison of the latter organism revealed more than 99% similarity to one of the "*G. hominis*" strains (207). It is obviously not possible to conclude how many species are involved, because all these rRNA similarity values are about 97% or higher and there is, at present, no other way to compare these isolates.

Although these names are probably only meant as working designations, they cause a lot of confusion, and therefore it is at present obviously more appropriate not to refer to such bacteria by the usual binominal species denomination. Murray and Schleifer (220) recently proposed that unculturable bacteria be included in a new category, *Candidatus*, pending a more thorough characterization and classification.

Population Genetics

The link of taxonomy with population genetics is essential to better understanding bacterial species determination. Population genetics deals with the variability of populations of bacteria and the formulation of theories that account for the variability. From studies conducted in this field during the last 20 years, the following findings are relevant for the present discussion and have been summarized by Maynard Smith (203).

Populations of bacteria consist of a number of independently evolving clones, a clone being defined as a set of genetically similar cells, derived from a common ancestor, without chromosomal recombination. It is of no use in bacterial taxonomy to try and formulate a "genetic definition" of a bacterial species, because recognized species clearly do not correspond to evolving populations that have a common gene pool, isolated from the gene pool from other species.

Clear evidence of recombination in bacterial populations is available from the mosaic structure of some nucleotide sequences, from the lack of association of alleles at different loci, and from the comparison of gene trees. In bacteria, limited local recombination occurs, giving rise to "mosaic structures" in sequence data as a result of horizontal transfer of pieces in the range of 10 to 1,000 bases only. Without recombination, the ancestral history of all chromosomal genes is identical and the phylogenetic trees derived from sequences of different genes should be similar.

There is no way of defining a species that will correspond to both a phenotypically recognized entity and an evolving unit and that will have, even approximately, the same meaning for all bacterial taxa. Maynard Smith (203) criticizes the bacterial species concept based on DNA hybridization for arbitrarily imposing divisions upon a continuum. Although a large number of generations can be easily observed in bacteria, the evolution of the bacterial genomes and thus the process of speciation are not well documented. A limited number of publications on experimental evolution (185) and on the general principles of the evolution of bacterial genomes (5, 6) is available. The science of experimental evolution might not only lead to fresh ideas on the bacterial species concept but might also eventually lead to the new field of experimental bacterial taxonomy.

Perspectives and Conclusions

One of the perspectives in bacterial taxonomy is that technological progress will dominate and drastically influence methodology, as it always has. More data will become available, more bacteria will be detected (whether they can be cultivated or not), there will be more automation, and software development will need to address the combination and linking of the different databases. We will also have increasing access to the genome, and numerous DNA sequences will be accumulating. The most challenging task will definitely be to process this mass of information into a useful classification concept. The different methods used in polyphasic taxonomy can be considered different windows through which the same landscape is seen. Polyphasic taxonomy is an attempt to synthesize the real landscape and a step toward a synthetic taxonomy which will be made possible through the development of new mathematical and information strategies. Although the idea is presently purely speculative, an insight into a vast amount of data could be the basis for a perfectly reliable and stable classification system. However, with our present data, it is already sometimes unclear whether it makes sense to order bacteria into a classification system. Undoubtedly, there is a huge amount of biodiversity, which can only be practical to handle if it is founded in an ordered structure, artificial or not, with appropriate terms for communication.

Large data sets should be numerically analyzed, and appropriate software packages can represent the results of the similarity comparisons as a two- or three-dimensional plot. In such plots, dense cores, each containing a different taxon, often seem to be present along with a number of intermediate strains. A classification system makes sense as long as this duality exists. The crucial question when we have access to growing data sets is the following: will this duality remain, or is it merely an image of our current limitations to study the full range of diversity, which will gradually disappear with the mounting piles of information?

It is worthwhile to attempt to summarize what we have learned about polyphasic taxonomy as it has been practiced during the last 20 years.

(i) The contours of the polyphasic bacterial species are less clear than the ones defined in the past by Wayne et al. (343) and take into account more elements than before. The bacterial species appears to be an assemblage of isolates which originated from a common ancestor population in which a steady generation of genetic diversity (5) resulted in clones with different degrees of recombination (according to the species), characterized by a certain degree of phenotypic consistency and by a significant degree of DNA-DNA hybridization and over 97% of 16S rDNA sequence homology. Although gene acquisition can obviously represent an interesting step in long-term evolutionary development of bacterial strains, it should not occur too frequently. This is necessary to respect the need for a reasonable degree of genetic stability, which is generally thought to be necessary to ensure the endurance of a species of organisms in ecosystems (6), whereas other results suggest a much higher genome plasticity (221). There is no phylogenetic standard for species, genus, or family delineation, nor is there a "gold standard" to identify bacterial species, and this is the major reason why a polyphasic approach is valuable.

(ii) A polyphasic approach to bacterial classification includes methods to phylogenetically allocate bacteria, methods to compare and group large numbers of strains into clusters of similar bacteria, DNA-DNA hybridizations to determine the relationships between representatives within and between each of those clusters, and descriptive methods which will provide further genotypic and phenotypic information. All possible methods that inform on the biological nature of strains merit equal attention in principle. Nevertheless, even the best laboratory can provide only a few sets of methods.

(iii) Polyphasic classification is purely empirical, follows no

strict rules or guidelines, may integrate any significant information on the organisms, and results in a consensus type of classification, satisfying most but not necessarily all users of taxonomic results. Polyphasic taxonomy is not hindered by any conceptual prejudice, except that the more information that can be integrated on a group of organisms, the better the outcome might reflect its biological reality.

(iv) The introduction of 16S rDNA sequences has definitively brought the phylogenetic dimension into bacterial taxonomy; its validation through the sequencing of other universally occurring genes is only a matter of time. The classical evolutionary tree may have horizontal shunts through which successful genetic information can be made available to other branches of the tree (5).

(v) Most bacteria in routine diagnostic laboratories will probably continue to be identified by means of classical methods, which are adequate, cheap, readily available, and easy to handle. For new or atypical isolates or in many research units where, e.g., bacteria are isolated from new sources, a straightforward identification of microorganisms by one single method is often not possible, and several methods are needed. The most direct approach is first to place such an isolate in the phylogenetic framework and then to determine its finer relationships by means of a polyphasic approach. This tendency of identification to become polyphasic is an unavoidable reality, and support of effective taxonomic reference laboratories is essential.

(vi) The automation, robotization, and development of databases will further develop, as is already the case for, e.g., the 16S rDNA sequence and fatty acid data. Ideally, there should be an international interconnection and accessibility of these databases. The necessity for reliability of the data is a conditio sine qua non. A recent initiative on integrated microbial databases has been taken by J. Tiedje, Michigan State University, East Lansing, Mich.

(vii) Some laboratories that have developed sequencing and fingerprinting technology also have access to computing facilities for storage and retrieval of fingerprints or sequences and similarity, correlation, or distance coefficient calculations and a variety of clustering techniques. It has become common practice to represent the outcome of the latter computations by means of dendrograms. Most of the computational techniques involved were developed in the 1960s and are designed for processing the data obtained by a single method. The comparison of different methods is complicated by the vastness of the data and the nonexistence of suitable objective methodology. The analysis and outcomes of different fingerprinting methods require the development of new methods of data fusion (or data aggregation). One of the solutions could be found, e.g., in the emerging theory of fuzzy logic (169), which is based on the simple yet powerful idea that an object is not forced to either belong or not belong to a given set but can have a partial degree of membership in this set. This would mean that bacterial strains do not have to belong to a single cluster.

(viii) Microbial taxonomy and biosystematics are a modern discipline that needs further financial and intellectual support to fulfil its role in diagnosis, biotechnology, and the study of biodiversity and the environment.

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