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CHAPTER 1.3

Defining Taxonomic Ranks

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Systematics and Classification

"Well, in *our* country," said Alice, still panting a little, "you'd generally get to somewhere else—if you ran very fast for a long time, as we've been doing."

"A slow sort of country!" said the Queen, "Now, *here*, you see, it takes all the running *you* can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!"

—Lewis Carroll, *Through the Looking Glass*, (1872)

Those who have chosen systematics, classification, and taxonomy as research topics have learned to consider the complexities as exciting and important. For others, these topics are mainly boring and through changes in names of microbial taxa, may cause confusion. Indeed, the problem of changing names of taxa is inherent. Classification is motivated by the desire of taxonomists to provide the user with a system that in their opinion optimally reflects the natural relatedness between the taxa. Now finally, the determination of phylogenetic relationships is achievable (Stackebrandt, 1992). Looking back in the history of microbiology, the lack of interest in classification can be traced to the enormous difficulty of past generations of systematists to put in order the phenotypic and genotypic properties of the steadily growing numbers of bacterial strains. The user of taxonomy was confronted with constantly changing classification concepts and systems, taxonomic rearrangements, and synonymy of names. Problems also arose from the terminology: while some regard systematics and taxonomy as synonymous, others define taxonomy as the theory and practice of classifying organisms and systematics as broader, including the evolutionary and phylogenetic components. For many researchers, nomenclature is their only contact with taxonomy, and the contact occurs only when they are confronted with name changes. However, systematics includes more than naming of organisms (Stackebrandt et al., 1999).

Classification is done by generating as much data on the properties of novel isolates as possible and by the process of identification, e.g., comparing the data with the database of previously classified organisms and by affiliating the isolate with a previously described or a new species. Classification includes the theory and process of ordering the characterized organisms into one or more systems. Nomenclature is the naming of the appropriate taxon within a classification system, and it includes subjective changes that occur whenever novel insights alter the taxonomic weight of characters, and thereby the rank of taxa.

As outlined (Stackebrandt et al., 1992), several classification systems exist in parallel and no classification system can claim predominance. No two systems of clustering need to match. As long as a system succeeds in doing what it sets out to do, it cannot be described as wrong or in error.

There are systems that group microorganisms on the basis of their increasing degrees of risk to humans, animals and plants. Here, organisms are artificially, pragmatically classified into risk groups according to degree of pathogenicity or risk potential, and this system serves no other purpose (Table 1).

Another system focuses on the rapid and reliable identification of bacteria for which knowledge about phylogenetic relatedness is not mandatory (Table 2). In such a diagnostic system, used in the past, affiliation of an isolate to a genus and species was based on Gram-stain reaction, oxygen requirement and morphology, chemotaxonomy, numerical phenetic analyses, usage of rapid diagnostic kits (e.g., API, Merieux, and BIOLOG), and combinations of selected physiological tests. (API System, La Balme les Grottes, 38390 Montalieu Vercieu, France. BIOLOG, Biolog, Inc., 3989 Trust Way, Hayward, CA, 94545 USA.)

Yet another system considers similarities in homologous molecules. Organisms are grouped according to their phylogenetic relatedness, which is then circumscribed by a wide range of genomic and epigenetic characteristics. This

Table 1. Examples of prokaryotic species classified by risk.

Genus	Risk group 1	Risk group 2	Risk group 3
Chlamydia	not known	*C. trachomatis*	*C. psittaci*
Bacillus	*B. circulans*	*B. cereus*	*B. anthracis*
Burkholderia	*Bu. cocovenenans*	*Bu. Cepacia*	*Bu. mallei*
	Bu. antropogonis	*Bu. Vietnamensis*	*Bu. pseudomallei*
Francisella	not known	*F. tularensis* subsp. *mediasiatica*	*F. tularensis* subsp. *tularensis*
Mycobacterium	*M. asiaticum*	*M. avium*	*M. leprae*
	M. fallax	*M. chelonae*	*M. tuberculosis*

Table 2. Examples of Bacteroidaceae classified by phenotypic properties.

Family	Genera	Main diagnostic differences
Bacteroidaceae[a]	*Bacteroides*	Peritrichous straight rod; produces a mixture of fermentation products from carbohydrate and peptone; butyrate not a major product
	Fusobacterium	Nonmotile straight rod; butyrate is a major product
	Leptotrichia	Nonmotile straight rod; lactate is the sole major fermentation product
	Butyrivibrio	Motile, not peritrichous, curved rod; butyric acid is the major fermentation product
	Succinimonas	Short, motile rod or coccobacilli; single polar flagellum; succinate and acetate are major fermentation products
	Succinivibrio	Motile helical or spiral-shaped cell; single polar flagellum; succinate and acetate are major fermentation products
	Anaerobiospirillum	Motile helical or spiral-shaped cell; bipolar tufts of flagella; succinate and acetate are major fermentation products
	Wolinella	Motile, helical to curved, or straight rod, single polar flagellum; either hydrogen or formate as electron donor for reduction of fumarate to succinate; carbohydrates not fermented
	Selenomonas	Motile, crescent-shaped cell, tufts of flagella on concave side; fermentation products are propionate and acetate
	Anaerovibrio	Motile curved cells, single polar flagellum, lipolytic; fermentation products are propionate and acetate
	Pectinatus	Motile curved cells, lateral flagella aligned on concave side; fermentation products are propionate and acetate

[a]Members of the family Bacteroidaceae are described as Gram-negative, fermentative anaerobic organisms (Holt et al., 1994).

genealogically based classification system is the most comprehensive one in overall understanding of the biology of the organisms, including the evolution of core processes of genetics, biochemistry, and physiology. This approach, which was outlined two decades ago, is applied by the vast majority of microbiologists.

Thus, classification in bacteriology is based on the principle of degree of relatedness. The grouping of organisms, whether or not they are formally designated taxa (e.g., genera, phyla, domains, groups, clusters, etc.), generally brings together those organisms on the basis of shared properties. Of the many systems that have been described in the past, the one that is based on genealogical relatedness offers the greatest potential, as it explains the widest range of genetic and biochemical properties. Genealogy derived from gene sequence similarity has the added advantage of working with a reliable, objective and stable basis for identification and classification. The dramatic changes that occurred in the classification of species of *Bacteroides*, lumped together in the past on the basis of a few superficial properties, are an excellent example of a shift to the concept that regards phylogenetic relatedness as the most reliable basis for classification (Table 3). The reclassification of a species requires redefinition of its properties. Analysis of the natural relatedness of a species will provide information on its phylogenetic position, i.e., its nearest neighbor(s). However, in many cases the position will not provide information on other properties needed to decide whether this species can be considered a species of a known genus or the nucleus of a novel genus. These conclusions depend upon the results of a wide array of phenotypic and genomic properties.

This chapter introduces the importance of gene and gene product sequence homology as a basis for an objective framework in which to order lineages of prokaryotic organisms. It then describes the (subjective) decision-making by which bacteriologists, on the basis of a phylogenetic framework, define the ranks of species and genera, and it deals with the problem of delineating ranks above the genus level. Special emphasis is placed here on the pragmatic definition of the species. An example of the process of

Table 3. Changes in higher classification as exemplified by the fate of some species of Bacteroides (see Table 2) after phylogenetic-polyphasic taxonomic analysis (Shah and Collins, 1989).

Traditional classification	Phylogenetic classification	Affiliation to higher taxon
Bacteroides furcosus	*Anaerorhabdus furcosus*	Bacteroidaceae
Bacteroides bivius	*Prevotella bivia*	Bacteroidaceae
Bacteroides endodentalis	*Porphyromonas endodentalis*	Bacteroidaceae
Bacteroides microfuscus	*Rikenella microfuscus*	Bacteroidaceae
Bacteroides amylophilus	*Ruminobacter amylophilus*	Gamma subclass of Proteobacteria
Bacteroides gracilis	*Campylobacter gracilis*	Epsilon subclass of Proteobacteria
Bacteroides hypermegas	*Megamonas hypermegas*	N.D.
Bacteroides nodosus	*Dichelobacter nodosus*	Beta subclass of Proteobacteria
Bacteroides termitidis	*Sebaldella termitidis*	N.D.
Bacteroides succinogenes	*Fibrobacter succinogenes*	Phylum Fibrobacter

Abbreviation: N.D. not determined.

modern classification is given for actinomycete taxa. This chapter should be studied in conjunction with the one by Wolfgang Ludwig (Ludwig, 1999), as many examples of lineages defined by 16S rDNA analysis, of the phylogenetic coherence or incoherence of taxa, and the delineation of higher taxa, can be judged best by having the phylogenetic tree available.

Past Classification Attempts

Ranks or taxa have been introduced in the classification of biological specimens to facilitate communication among men and to arrange living matter by morphological, physiological, ecological and genomic features. The basis of any system is the species and the genus, and according to the binomial system (Linnaeus, 1753), the description of a type species is not possible without describing a genus, and a genus cannot be described without a species. This simplicity explains why the binomial system is still used for the naming of organisms within the three domains Archaea, Bacteria and Eucarya (Woese, 1987). Moreno (1997) states: "The wisdom of Linnaeus was not only to create a comprehensive classification system, but more importantly, a useful one." The definition of a species has been debated extensively since the publication of the key work—*On the Origin of Species* (Darwin, 1859). At that time, the debate centered on animals and plants but excluded the prokaryotes mainly because they had no evolutionary record.

Bacterial classification as a science began with the contribution of Cohn (1872, 1875), who was the first to ask if bacteria, like plants and animals, can be arranged in species and genera. He presented a classification scheme composed of six genera that were distinguished on the basis of morphological criteria. However, he clearly pointed out that morphological properties are insufficient, inasmuch as similarly shaped bacteria may have different physiological characters. Cohn regarded the genera as natural entities but the species he described as largely provisional. With hindsight, it is possible to conclude that early microbial systematists were in no position to judge the importance of such simple properties in evolutionary terms. It was not known until the late 1970s that taxa defined by superficial properties such as morphology did not necessarily match taxa defined by traits that arise in the course of long evolutionary processes.

In the early twentieth century, the number of determinable properties expanded dramatically and, consequently, the number of species increased. New strains of medical importance were described as new taxa and the classification systems placed emphasis on these organisms and their identification. New systems were proposed in which the accent shifted from morphology to physiology, metabolism, pigments, and pathogenicity (Migula, 1900; Orla-Jensen, 1909; Pringsheim, 1923; Prévot, 1938; Kluyver and van Niel, 1936; Stanier and van Niel, 1941). To incorporate the wealth of information and to integrate the different prokaryote systems with those of higher animals and plants, a single unified formal system of bacterial classification was established by Buchanan (Buchanan, 1916, 1918). This system provided the basis for *Bergey's Manual of Determinative Bacteriology*, which, in the many editions that followed, presented better than any other source the most useful references for identification but retained a nomenclature that connoted phylogenetic relationships, in the tradition of Buchanan's system. Attempts to construct another single formal classification system or to work with several systems in parallel were criticized. Kluyver and van Niel (1936) suggested that rather than searching for a natural system, taxonomists should develop determinative keys to provide the easiest possible identification of species and genera. However, because the choice of characters used to establish the system was subjective, it was recognized that empirical sys-

tems would be largely unmodifiable. Consequently, the whole system was disrupted when novel characters were taken as the basis for the establishment of a new classification system. The main advantage of the empirical system was its immediate practical utility, but even this advantage disappeared when differential characters were not actually mutually exclusive. The period during which the importance of developing a natural classification system was recognized (Stanier and van Niel, 1941) but considered unachievable lasted until 1975. The question then remains why past generations of microbiologists could not develop a phylogenetic framework for prokaryotes. In hindsight, the answer is quite easy: Early attempts were prone to failure because scientists lacked fundamental genetic information, understanding of mechanisms of heredity, and the technical ability to find out the structure of genes and chromosomes.

Phylogeny Is Based on Homology

Phylogenetic systematics seeks congruence between the lines of descent evolved over time and the supraspecific taxa described by taxonomists. Prerequisite for the description of a taxon of any rank in a phylogenetic system is the recognition that all members originate from one ancestral form and that homologous traits of the ancestral form are found also in their descendants. The question then remained which of the several thousand semantides in a prokaryotic cell are useful for phylogenetic studies. The establishment of a system which is set up to subsume all species must include phylogenetic markers that are ubiquitously distributed, functionally equivalent, and homologous housekeeping molecules. These markers should be homologous apomorphic characters that evolved only once (synapomorphy) but not by convergence. Homology is the sharing by two taxa of a property derived from the same or equivalent property of the nearest common ancestor. Deciding whether a property is homologous or the product of convergence has been the greatest problem, and one that could have been solved only by comparing the course of evolution of each property as laid down in the fossil record. It is obvious that lack of a substantial fossil record and thus the inability to use that record to draw conclusions on genomic and phenotypic properties have prevented the inductive derivation of genealogical lines. Furthermore, morphological complexity and comparative anatomy, extremely useful properties of eukaryotes for determining homologies, are absent in the morphologically and developmentally simple prokaryotes. As a consequence, a phylogenetic classification system only became available after the theoretical and methodological basis had been laid about 30 years ago.

One of the main intellectual breakthroughs that helped microbiologists solve the problems of phylogeny was provided by Zuckerkandl and Pauling (1965), who recognized that organisms are the products of historical events and that all cellular structures reflect their evolutionary history. These scientists also commented that in the case of microorganisms, early evolutionary events can be documented only at the primary structural level of homologous and phylogenetically informative molecules. When comparing organisms, the number and composition of sequence differences between corresponding proteins and genes coding for rRNA reflect phylogenies and consequently allow the recognition of pairs or groups of organisms that originated from a common ancestor. Determining relatedness is based on sequence analysis of genes or their transcripts, also known as the semantides.

There are three categories: primary (DNA), secondary (RNA), and tertiary (proteins) semantides. Sequences of these molecules are molecular chronometers, records of evolution, as they indirectly measure the time lapsed since their origin, and the comparative analysis of primary structures is a powerful tool to measure evolutionary relationships. Episemantic molecules to be used in comparative studies are synthesized under the control of tertiary semantides, and above all it is the chemical composition of cell constituents that have received considerable attention (Schleifer and Kandler, 1972). Asemantic molecules (e.g., exogenous vitamins, phosphate ions, oxygen, viruses) are not produced by the organisms themselves and do not express any of the historic information that organisms contain.

Zuckerkandl and Pauling state that "at any level of integration, the amount of history preserved will be the greater, the greater the complexity of the elements at that level and the smaller the parts of elements that have to be affected to bring about a significant change. Under favorable conditions of this kind, a recognition of many differences between two elements does not preclude the recognition of their similarity." The correctness of this hypothesis was demonstrated by the impressive phylogenetic trees of gene and protein sequences. Episemantic molecules were not considered useful for deriving evolutionary conclusions because enzymes with different primary structures can lead to the synthesis of identical episemantic or similar molecules in different organisms as long as the active enzymatic sites are similar.

The Main Phylogenetic Parameters for Classification

The two main tools [sequence analysis of the semantides DNA, RNA and proteins (Zuckerkandl and Pauling, 1965) and hybridization of genomes] for determining phylogenetic relationships in prokaryotes were developed in the mid-1960s. Historically, the molecular approaches used were sequence analyses of proteins, such as cytochrome C, fibrinopeptides, and ferrodoxins, as well as immunological approaches, such as immunodiffusion and microcomplement fixation. However, the latter methods as well as protein sequencing became less significant with the availability of rapid sequencing techniques for DNA. The branching patterns based upon 16S rRNA and 16S rDNA sequences were surprising, mainly because these patterns showed that characters used traditionally to cluster organisms have in reality restricted phylogenetic meaning. Prominent examples of phenotypic characteristics shown not to circumscribe higher taxa in the past are now examples of characteristics whose evolutionary development has recently been or is soon to be unraveled. These are cell walls (Kandler and König, 1985; Stackebrandt et al., 1985; Schleifer et al., 1990), aerobic metabolism (Fox et al., 1980; Seewaldt et al., 1982), spore formation (Ash et al., 1991; Collins et al., 1994; Stackebrandt and Rainey, 1997), biosynthetic pathways (Balch et al., 1979; Fowler et al., 1986; Stackebrandt et al., 1989), and photosynthesis (Gibson et al., 1985; Woese et al., 1985a; Stackebrandt et al., 1988; Imhoff et al., 1998a; Imhoff et al., 1998b). Today, sequencing of 16S rDNA in bacteriology is so widely accepted that it is considered a classical approach. The sequencing and analysis strategies and their bearing on evolution, phylogeny, classification and identification are now textbook knowledge.

16S rDNA Analysis

Though the importance of rDNA sequencing, alignment, and data analysis is covered extensively in chapter 1 (Ludwig, 1999), the main points are repeated here. The primary structure of the rDNA molecules spans an enormous geological period, almost 3.6 Gy. Why especially has this molecule been selected for phylogenetic analysis? Both the gene and its product are ubiquitous and have functional constancy, common ancestry, genetic stability, appropriate size, and independently evolving domains within the molecule.

Sequence analysis of rRNA or other phylogenetically meaningful genes (Schleifer and Ludwig, 1989) has become a rapid standard technique, and the sequences generated have a very low error rate. The restriction in the use of this molecule as a phylogenetic marker springs from certain intrinsic properties: considering the billions of years that have passed since their origin, the number of informative positions within sequences is small. To maintain function, a reasonable percentage of the positions must remain invariant or be highly conserved, and many of the remaining characters cannot be changed independently. As a consequence, the majority of evolutionary events will remain undiscovered. Another restriction is that most prokaryotic organisms have 2–14 copies of the multigenic rRNA operon in the genome (Farrelly et al., 1995). PCR amplification will mask possible intracistronic microheterogeneities, which consequently may obscure elucidation of small differences between closely related organisms. Sequence analysis of individually cloned operons will unravel these heterogeneities but these few changes must be regarded as "noise" and hence without phylogenetic implications.

Higher order structures of rDNA and rRNA molecules facilitate sequence alignment which can easily be done manually. Several algorithms are available for phylogenetic analyses (Ludwig, 1999). Once a phylogenetic tree or a dendrogram is generated, the taxonomist must judge whether the branching order of the phylogenetic tree is reliable. Numerous factors can influence the topology of a branching pattern, which is a dynamic construct that changes with any new sequence included, or region selected, for analysis. Nevertheless, the topology of trees, which are generated on the basis of genes subjected to the same fate in evolution (e.g., common horizontal or vertical gene transfer), are rather stable and robust constructs. Results of comparative analyses of other conservative molecules responsible for central functions such as the β–subunit of ATP synthases, elongation factors, phosphoglycerate kinase, and DNA-directed polymerase demonstrate this point. Thus, trees based on rDNAs and rRNAs reflect not only the evolution of these molecules but also, most likely, the evolution of a major portion of the genome. In principle, the primary structure of the most widely analyzed 16S rRNA gene must be regarded as a miniaturized version of a major part of the genome, and, though due to its size of only 1,540 bases, as having much less power to resolve.

DNA-DNA Hybridization

This method was the first molecular approach used routinely for measuring degrees of relatedness and the first phylogenetic one to be generally accepted for improving bacterial clas-

sification. It is still the most rapid and inexpensive of all phylogenetic methods for measuring an average nucleotide similarity of the entire genome; however, it gives no indication of which genes contribute to or detract from the similarity. Also, this technique reveals why neighboring species show somewhat lower similarity values. Though unclear and unsatisfactory, the information found in a genome, containing a range of highly to less conserved genes, has the advantages over information obtained by comparison of individual genes or gene products only. Several hybridization techniques have been thoroughly tested to determine the influence of various experimental parameters and compared to determine reproducibility and limitations (Grimont et al., 1980; Huss et al., 1983; Baumann et al., 1983; De Ley, 1970; De Ley, 1970; Schleifer and Stackebrandt, 1983). DNA-DNA pairing studies, comparing the same strains by different techniques, were in good agreement (see Schleifer and Stackebrandt, 1983 for examples). Some novel techniques and variations of established methods have been introduced such as hybridization in microdilution wells (Ezaki et al., 1989; Hara et al., 1991; Kaznowski, 1995), the random-primed labeling and signal amplification system (Amersham Life Science, Piscataway, NJ), or detection of double-stranded, digoxigenin-(DIG) labeled DNA with anti-DIG antibodies conjugated with alkaline phosphatase (Lind and Ursing, 1986; Ziemke et al., 1998). Some of these novel methods have not been compared with the established ones (S1-, renaturation- and filter methods), but others such as the microplate technique (Ezaki et al., 1989) and the renaturation technique correlated very well (Goris et al., 1999).

Relationships are usually expressed in terms of DNA similarities. It should be noted that because the underlying processes of renaturation are still unknown, the expression "DNA homology" should not be used in connection with DNA reassociation techniques. Wayne and colleagues (1987) recommended use of a second parameter, the Tm(e) value (Tm(e) is the melting point of DNA formed originally by the reassociation process of two single stranded DNA molecules; (e) stands for eluted labeled single stranded DNA which is released by heating up the double stranded DNA), especially in those cases where, under optimal hybridization conditions, DNA similarities fail to discriminate between fine details in relationships. The inverse linear correlation between Tm(e) and DNA similarity makes determination of both parameters somewhat redundant (Grimont et al., 1980; Baumann et al., 1983), and hence Tm(e) values is usually not included in DNA-DNA reassociation studies.

Greater reproducibility and small sampling error (Sneath, 1989) are obvious advantages of DNA hybridization. The disadvantages are the unavailability of equipment, i.e., a thermocontrolled spectrophotometer, and lack of DNA in sufficient quantity and purity, as exists with many archaea and lithoautotrophic bacteria. The limited resolving power of DNA hybridization has been recognized from the very first experiments. It has been calculated that for reassociation under optimal hybridization conditions (25°C below the Tm(e) of the DNA), the two DNA strands must exhibit at least 80% sequence complementarity. Depending on the sequence similarity of the reassociating single strands, a difference of about 20% is then spread between 0% (no hybridization) and 100% (as defined by maximal reassociation obtained with the homologous DNA strands). It is therefore obvious that a given DNA homology value does not reflect the actual degree of sequence similarity of the primary DNA structure. As measured with experimentally introduced mispairings, thermal stabilities have been estimated to decrease from 1 to 2.2% for each percent mispairing (Bautz and Bautz, 1964; Britten and Kohne, 1968; Ullman and McCarthy, 1973). Although these experiments have been performed on short stretches and not on complete genomes, one can nevertheless argue that organisms that share 70% DNA similarities share at least 96% DNA sequence identity (Johnson, 1973). If the number of bases in the genome of E. coli is approximately 4×10^6, then 4% differences or 1.6×10^5 nucleotides are different (not taking into account the possibility that genome rearrangement is a source of decrease in DNA similarity). This divergence could easily account for the significant differences in phenotype observed between strains of some species, e.g., *E. coli* (Brenner, 1991).

Correlation of Individual Phylogenetic Parameters

It is generally accepted that if two organisms have highly similar DNA, they are closely related genetically. The parameters measured by different methods have shown excellent agreement when closely related organisms are compared. However, when parameters from more distantly related organisms are measured, the data are difficult to reconcile (Huss et al., 1983; Grimont et al., 1980). Numerous studies have shown that phenotypic and genetic similarities agree only if the borderline of 70% similarity is obtained under optimal hybridization conditions. Therefore, this borderline has been recommended for species differentiation (Wayne et al., 1987). Values from 30 to 70% reflect a moderate degree of relationship, whereas values become

increasingly unreliable (and taxonomic conclusions should be avoided) once these values fall below the 30% level. One has, however, to consider that this recommendation was derived mainly from experience of working with numerous strains of enterobacterial species (Steigerwalt et al., 1976; Brenner, 1991). Thus, transferring the situation found for a phylogenetically very shallow group of mainly eukaryote-associated organisms to two ancient, highly structured, and enormously diverse prokaryotic domains grossly underestimates the different mechanisms as well as the mode and tempo by which organisms evolve. But then one has to remember that the delimitation value (of 70%) is artificial and used to structure the bacterial world at the level of species.

For highly related organisms, there is very good congruence in general between DNA-DNA and DNA-rRNA hybridizations (De Smedt and De Ley, 1977; De Vos and de Ley, 1983; Johnson and Francis, 1975). The Tm(e)s for strains of species exhibiting more than 60% DNA homology differ by less than 2°C. However, the DNA-rRNA hybridization technique has been superseded first by 16S rDNA cataloguing and then by sequence analysis of 16S rDNA. Values on DNA-rRNA hybridization are found in the literature before 1995. Likewise, similarity coefficients of the 16S rRNA cataloguing approach (S_{AB} values), the predecessor technique of 16S rDNA similarity determination, are found in the literature before 1990. For the same reasons as for total 16S rDNA, correlation between DNA pairing values and S_{AB} values is only marginal (Stackebrandt, 1992). Only of historic value are the correlation blots between rRNA homology and S_{AB} values (Schleifer and Stackebrandt, 1983) and the correlation between S_{AB} values and actual almost complete 16S rRNA sequence homologies (Woese, 1987).

The correlation blot, determined for the two most widely used approaches for discovering prokaryotic phylogeny, justified continuing the use of the DNA-DNA reassociation technique. Let us assume the unlikely case that the plot showed linear correlation between intraspecies DNA similarities of above 70% and 16S rDNA sequence similarities above 97.5%. The DNA hybridization method would have disappeared overnight. Unfortunately (for those who are using the reassociation technique) the situation is different. The 16S rDNA is not a miniaturized mirror image of the genome but is too constrained by its function to change as quickly as less conserved molecules. As a consequence, there is a curvilinear relation between the two parameters (DNA-DNA reassociation and rDNA similarities; Amann et al., 1992; Fox et al., 1992; Stackebrandt and Goebel, 1994). Each approach is strong in those relationship areas that other methods are weak in. Sequence analysis has proven to be a reliable way to distinguish the phylogeny of organisms of different domains (with 55–60% similarity) from moderately related species (around 97% similarity). Above 97% 16S rDNA hybridization values can be as low as 55% or as high as 100%. Several organisms, which are known to share 99.8% or even 100% rDNA similarity, belong to different species because the DNA reassociation values are below the 70% threshold value. Even if one considers that the DNA reassociation values originated from different laboratories using different reassociation methods, the evidence is strong enough to state that the sensitivity of DNA-DNA reassociation is significantly greater than that of 16S rDNA sequencing. When a 16S rDNA similarity value of less than 97% was found to correspond to a DNA-DNA reassociation value of not more than 60%, Stackebrandt and Goebel (1994) recommended that DNA pairing studies did not have to be performed at this and lower levels of sequence similarity. These levels indicated that the strains concerned are not members of the same species.

The correlation blot of 16S rDNA and DNA-DNA similarity values obtained for some species described in 1998 and their nearest phylogenetic neighbors (Fig. 1) demonstrates that the recom-

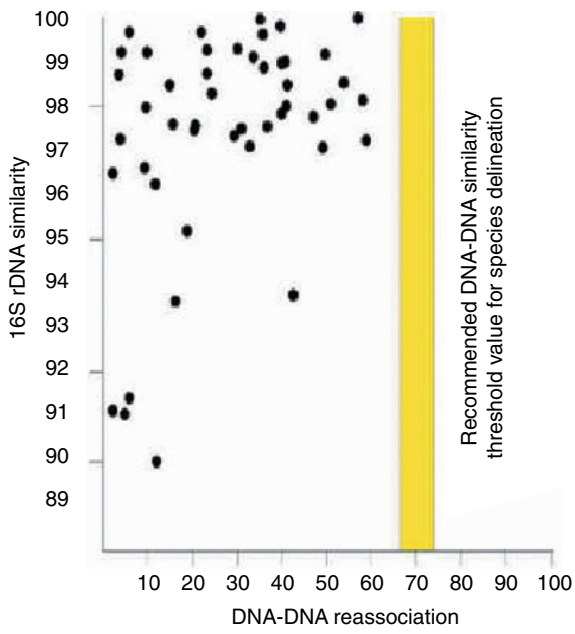

Fig. 1. Correlation blot between 16S rDNA and DNA-DNA reassociation similarities, indicating for several phylogenetically highly related species (as determined by 16S rDNA values of >98%) that DNA-DNA similarities are clearly below the threshold value of 70% recommended for species delineation. Values are taken from volume 48 of the *International Journal of Systematic Bacteriology* (1998).

mendation of Wayne et al. (1987) has been accepted by taxonomists. Except for a single case, all strains of a species share higher than 70% DNA-DNA similarity, while they share less than 70% DNA-DNA similarity with their nearest neighboring species. The one exception refers to *Pelistoga europaea*, which have four genomovars that are related at the 40% DNA similarity level. The authors (Vandamme et al., 1998), however, clearly state that the lack of phenotypic evidence presently excludes the description of four individual species, a step that may be necessary once these differentiating characteristics become available.

Comparison of Phylogenetic Patterns of 16S rDNA

The increasing number of dendrograms and phylogenetic trees in the literature reflects the common notion that the 16S rDNA sequencing method is a "traditional" method. Many trees are not comparable as they were generated from partial sequences and different treeing methods (summarized by Felsenstein, 1982, 1988) and are therefore of historic interest only (Stackebrandt, 1988, 1992). Today nearly complete sequences are compared using a few treeing algorithms of proven resolving power and statistical significance (Ludwig, 1999). But some problems remain. First, the parts of the sequence judged to be of less phylogenetic importance are subjectively omitted. Second, computer programs cannot handle the enormous amount of data in a reasonable time without omitting either the number of reference organisms or sequence information. However, one must differentiate between goals; this determines the number of sequences used. For taxonomic studies, e.g., questions about the intrageneric relationships, the number of sequences is mostly restricted to those of type strains and a few others. In this case the complete sequence information, including that of the variable regions, can be compared. The branching pattern obtained will change if this small dataset is embedded in a larger one composed of sequences of members of families, orders, classes, and so on. At each level, information will be lost by either removal of variable regions or trimming of stem and loop structures to the minimum length common to all members of the dataset and by omission of those regions for which ambiguous sequence information is provided. Each of these steps will most likely lead to changes in the branching pattern of any lineage. Thus, the picture created from the inclusion of thousands of sequences in a single dataset is not more than an approximation of the phylogeny. The literature is full of examples that demonstrate changes of phylogenetic relatedness within genera and families through the influence of new entries in the database. Most of the branching patterns are supported by high bootstrap values that for a given tree indicate that the statistical analysis supports the order of lineages. But this statistical analysis is per se no indication that the pattern reflects the natural relationship with a similar degree of confidence.

If an algorithm does not exist that could provide a tree more closely reflecting the evolution of prokaryotes, then we must accept the pattern that appears to be the most plausible one. It may be the one showing the highest degree of topographic similarity to patterns derived from different informative molecules, although it should be noted that these similarities are not per se proof of phylogenetic "truth." The phylogenetic framework referred to as the most convincing one for the bacterial and archaeal kingdoms is today classified as domains Bacteria and Archaea (Woese, 1987; Olsen et al., 1994).

The Prokaryotic Species: A Natural Entity or a Taxonomic Myth

The evolutionary record as a basis for phylogenetic studies can now be found in the extensive database of molecular sequences, which have placed the bacterial world into the framework of the evolutionary process. But this information alone does not help describe a species or how a species has to be defined. Rather, it puts the prokaryotes on the same level with animals and higher plants in the debate about species as a general unit for biodiversity, evolution and taxonomy. Does this mean that biologists can now define the category species as a comparable biological entity for every organism? The problem is that biologists themselves are not clear about the definition of "species," a concept that lacks a theoretical basis (Bachmann, 1998). The concepts of phylogenetic species (Cracraft, 1983), taxonomic species (Staley and Krieg, 1984), biological species (Dobzhansky, 1937; Istock et al., 1996), which disregards asexual reproduction entirely, and ecological species (Istock et al., 1996) have strengths and weaknesses, and each of them stresses different aspects of biology and evolution. In his dictionary of microbial taxonomic usage, Cowan (1968) even states bluntly that the species is not a natural entity. Surprising perhaps to microbiologists, there are some zoologists (Hull, 1997) and botanists (Bachmann, 1998) who suggest the possibility that the species is not an objective basic unit of taxonomy. The nonexistence of species as an objective category and as a product of natural selection, which after sufficient study is identifiable by the taxonomist, has been recognized by microbiologists for more

than 20 years. Bacteriologists in particular follow guidelines and recommendations to provide stability, reproducibility, and coherence in taxonomy—although in the final analysis, species description is still subjective. This concept does not include the role of reproductive isolation, i.e., the barriers to horizontal gene transfer over large phylogenetic distances; it does not even try to explain the mode of speciation. One may be amused by such a naïve approach—but once you decide that a species can not be recognized as a natural entity, the only alternative is a compromise of a working definition. In that, the fundament of a species can be found in genealogical relatedness of its members (Wayne et al., 1987; Stackebrandt and Goebel, 1994). This strategy has facilitated the practice of taxonomy—a strategy also used by protozoologists, mycologists and algologists. As Bachmann (1998) points out, the most useful general species definition would be the one that allowed "the largest number of individual organisms to be unequivocally assigned to species so that some basic conditions are satisfied." These conditions are: 1) strains are assigned to only one species and never to none; 2) all lines of descent within a species are members of that species; 3) members of a species should be phylogenetically related; and 4) the species (so defined) should apply to taxa that coincide more or less with the intuitively recognized species. Most obviously, conditions 1) to 3) are already in effect in bacteriology, while condition 4) has failed significantly in the past because of inability to classify a prokaryotic species by intuition.

There is no recognized concept of a prokaryotic species, though Istock et al. (1996) defines nine different mechanisms for their evolution. Nevertheless, the pragmatic definition is well accepted among bacteriologists. The combination of phylogenetic grouping based on sequence comparisons with taxonomic classification is a very powerful approach. This polyphasic approach is the only accepted strategy, which does not mean that certain components of the approach are not criticized. The process of revision and constant adaptation appears necessary as not only new insights into cell structure and cell function should be incorporated but also the microbiologists' perception. As Staley and Krieg (1984) phrased it: "A classification that is of little use to the microbiologist, no matter how fine a scheme or who devised it, will be ignored or significantly modified." This is true for each level within a hierarchical system, and the history of microbiology is marked by many examples of rejected systems. The higher ranks are almost completely defined by subjective arguments—to the point where the importance of working with taxa above the rank of genera is considered trivial (O'Hara, 1994). The importance of any hierarchical system goes beyond the main function of classification and identification. Based on knowledge available at the time of its establishment, a hierarchical system should explain and increase understanding of the evolution of organisms and their groupings. In bacteriology, time has seen various hierarchical systems and various proposed phylogenetic paths fail because they were not based upon the natural relationships but rather on properties believed to express natural relationships such as morphology (Cohn, 1872; Stanier and van Niel, 1936), pigmentation, physiology (Orla-Jensen, 1909; Margulis, 1981), and cell constituents (Schleifer and Kandler, 1972). Some of these attempts were important contributions in their time because the classification system based upon them actually reflects phylogenetic divergence (e.g., peptidoglycan structure, lipids, fatty acids). This strategy has changed now, as the order of phylogenetic lineages guides the bacteriologists to the two basic units, the genus and species, without the need for a superimposed system. Actually there is no immediate need to work with a hierarchical system, but it is tempting to do so to comprehensively classify similarities, differences and evolutionary traits. Time and the recognition of the semantic character of the macromolecules DNA, RNA and proteins have shown that this basis was lacking (Zuckerkandl and Pauling, 1965). Today, we see the emergence of higher taxa along the phylogenetic structure and, like in systems of plants and animals, taxa of the same rank are not necessarily comparable units or describable in a coherent way. Also, we must be aware that only a small fraction of prokaryotic species are described, and new entries will not only change the description of the higher ranks but may change the composition of taxa as well. However, the advantage of a rational hierarchical structure, i.e., based on the organisms' evolutionary history, makes it highly likely that changes within the system will occur only within ranks of a common genealogical lineage and not, as in the past, affect and possibly change remotely related taxa.

The species definition applied today does not incorporate the modus of grouping entities into named "natural" species. Several factors that contribute to the evolution of the genome have been identified through intensive multilocus enzyme electrophoreses and sequence typing of housekeeping genes (Maiden et al., 1996) and of random amplified polymorphic DNAs and multilocus enzyme electrophoreses (Selander et al., 1994; Istock et al., 1996). Some organisms, e.g., *Neisseria* and *Rhizobium* species, as well as enterobacterial species (Guttmann and Dykhuizen, 1994), are subjected to reticulate events or

panmixis (Maynart-Smith, 1993; Istock et al., 1996) in which clonal relationships, due to mutational events and vertically transmitted accessoric genetic elements, are disturbed by horizontal genetic transfer, e.g., conjugation, phage transduction DNA transformation (Achtman, 1998). Other strains that are mostly endosymbionts and obligate pathogens, such as members of the genera *Bartonella*, *Brucella*, and *Rickettsia*, are mainly clonal because they are subject only rarely to horizontal gene transfer. In some species the recombination is more frequent among strains of different than of the same species (e.g., the enterobacteria), which leads to the homogenization of the gene pool of the interacting organisms (Guttmann and Dykhuizen, 1994). An attempt to formulate a biological species definition for bacteria takes the following observations into account (Dykhuizen and Green, 1991): 1. Phylogenetic trees from different genes from members of a single species should be different (shown for three genes from *E. coli*). 2. Phylogenetic trees from different genes from members of different species should be the same (as shown for two genes from seven species of *Neisseria*). Without questioning the validity of this approach, it is obvious that this strategy is far beyond the capability of routine sequence analysis methods, especially inasmuch as several strains of a single species must be investigated, and although worth discussing, this approach cannot immediately replace the present pragmatic species definition.

The current pragmatic species definition also does not account for the ecological niche, although the source of the isolate is part of a species description. This site is the strain's actual place in the ecosystem provided the strain is dependent on its environment, e.g., the rhizoplane, rhizosphere, and host in endosymbiotic and pathogenic relationships. The terms (marine water, fresh water, mud, sediment, soil, rumen, skin and so on) are too superficial to describe the exact niche from which complex environmental samples are taken. Knowing about the site of speciation and the environmental selection of members in a clonal population may help explain the path of evolution and the mode of speciation, but this information does not help define the level at which a subpopulation may be regarded as an individual species.

The Pragmatic Species: Definition

The definition of a prokaryotic species has a phylogenetic component given by Cracraft (1983) as "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descendents" and a taxonomic component given by Colwell (1970) as "a group of related organisms that is distinguished from similar groups by a constellation of significant genotypic, phenotypic, and ecological characteristics." This definition combines descriptions of geno-, or genomospecies, taxospecies, and phenospecies, which reflect the different concepts of species upheld in the past decades. From a pragmatic point of view, all these facets have been incorporated into a single definition, though the terms are still in use. An "optimal" species is one that simultaneously represents a phylogenetically, phenotypically, and naturally occurring group, but except for many strains of pathogenic species, the species' ecological niche is either not known or the number of isolated strains is too small to identify their original habitat. It should be remembered, that from the broad diversity of prokaryotic organisms, which may reflect a genetic and epigenetic continuum, a single strain is chosen as the type strain. Strains that are sufficiently similar, i.e., by mainly DNA-DNA reassociation used today in prokaryotic taxonomy, are considered members of this species. This concept of selecting species has been described as the arbitrary species concept (Staley, 1997). The combination of arbitrary selection and artificial species delineation is admittedly arguable and open to discussion, especially when taxonomists covering different fields of biology meet. When compared with the phylogenetic diversity of a prokaryotic species, the phylogenetic diversity of *Homo sapiens* and its closest relatives, the higher evolved apes (all species from man to lemurs, comprising about 200 species, which are related by higher than 75% DNA reassociation; Sibley et al., 1990), would be within the 70% threshold value. Obviously, the species definition of prokaryotes cannot be applied to eukaryotic organisms. For prokaryotic organisms the pragmatic approach to the species definition has been extremely useful and its success is measured by its widespread acceptance.

Delineating a Species

In the daily routine a new isolate runs through an identification process, which may be different from laboratory to laboratory and from taxon to taxon. Many scientists, however, are not interested in a fine resolution of relationship, or they are not in a position to go through the laborious identification process. Some initial superficial tests are performed, like determination of colony morphology and pigmentation, shape, spore formation, Gram stain and relationship to oxygen. The aim of the study is to define the identification procedures to follow—but it can be

expected that phylogenetic diversity in many similar-looking organisms remains undetected. The classification process starts when the isolate does not fit the description of one of the 4,200 validly described species.

Let us assume the laboratory is equipped with facilities to generate a 16S rDNA sequence. It is recommended to start any survey with this molecule, as the database of prokaryotic strains is enormous, covering more than 95% of described species. To search for the closest relative using available 16S rDNA sequence data, taxonomists are offered electronic help by the BLAST system (blast.help@ncbi.nlm.hih.gov), the Ribosomal Database Project (http://www.cme.msu.edu/RDP), or updates of the ARB program (http://www.arb-home.de). The taxonomic browser will guide the user to a phylogenetic arrangement of taxa. The search in RDP and ARB will show the phylogenetic distance to the isolates' nearest neighbor(s), but the quality of the search depends upon the completeness of the 16S rDNA database. The sequences available, species analyzed, and whether comparison is based on short stretches or on almost complete sequences, may vary. Once the approximate nearest phylogenetic neighbor has been identified, a search of the public databases for recent entries is recommended.

The result of the analysis will affect future strategy. Let us assume that the 16S rDNA similarity to its nearest neighbor has values higher than 97–98%. Many scientists will be satisfied knowing the approximate phylogenetic position and will not continue the identification process. Others, however, will be eager to determine the more precise affiliation of the isolate. The way to proceed depends upon the number of species in the phylogenetic vicinity of the isolate.

1. In case the isolate falls within the boundaries of a genus, the description of this genus will be a guide to the few key properties needed to place the isolate in this genus. If the species within this genus are separated by distinct phenotypic properties (which one should assume but which is not always the case), these should be sought in the isolate and, if present, the isolate has been identified. If not, DNA-DNA reassociation studies are recommended to determine whether the isolate is the nucleus of a new species. In case DNA similarities are lower than about 70% DNA-DNA reassociation, the isolate should be described thoroughly, providing evidence for the genus characteristics and those properties that distinguish the new species from the established ones.

Examples are found frequently in the literature and a few of them are shown here:

The most widely encountered situation is the description of a new species which shows less than 70% DNA-DNA reassociation with those species to which it is closely related by 16S rDNA similarity: Hybridization values for the type strain of the new species and that of the closest relative usually range between close to 70% and almost zero percent. To give two examples, *Kocuria rhizophila* is separated from *Kocuria varians* by a similarity value of 52.6% (Kovacs et al., 1999), while the DNA-DNA relationship between the type strains of *Methanococcus infernus* and *Methanococcus jannaschii* was less than 10% (Jeanthon et al., 1998).

In those cases where the DNA-DNA reassociation value between an isolate and a described species is ≥70%, this information will usually not be recorded, unless the new strain leads to the description of a subspecies or to an emendation of the species description. Many examples exist in the literature that demonstrate the intraspecies genomic homogeneity.

DNA-DNA reassociation reveals that the type species of two different species are actually strains of the same species: The species *Kocuria erythromyxa* was reclassified as *Kocuria rosea* on the basis of 95% DNA-DNA reassociation and similar phenotypic differences (Schumann et al., 1999).

Species are separated at the threshold value of around 70% DNA-DNA similarities on the basis of differences at the epigenetic level. For example, the separation of the type strains of *Desulfurella acetivorans* from *Desulfurella multipotens*, sharing 69% DNA-DNA similarity, was based on the ability of the latter strain to use butyrate as growth substrate and to grow chemolithoautotrophically on mineral medium containing molecular hydrogen, CO_2 and elemental sulfur (Miroshnichenko et al., 1994).

The recommendations (Wayne et al., 1987) to delineate species in genomic terms at a threshold value of around 70% DNA-DNA reassociation are guidelines but should not be applied as fixed rules. Though the majority of species are actually described as suggested, there are a few exceptions:

One example refers to the lack of any phenotypic differences in the two putative species that are separated by higher than 70% DNA-DNA reassociation. In practice, it would be impossible to affiliate other strains to either species and to distinguish between the two species without performing DNA-DNA reassociation studies (Vandamme et al., 1998). One should, however, consider that the primary structure of a 16S rRNA, like that of other genes, is a linear compilation of phenetic characters, whose composition at a defined position should be treated as a phenetic property. For example, ornithine at position 3 of the peptidoglycan subunit is treated as a different taxonomic marker than the pres-

ence of diaminopimelic acid or lysine at the same position in a different organism. Thus the differences in nucleotides involved in compensatory base exchange in the 16S rDNA nucleotide sequences that occur between two closely related species distinguishable by DNA-DNA reassociation should be accepted as characters with discriminating power. Using this strategy, a new species of *Sulfitobacter mediterraneus* has been described which by the profile of its metabolic properties could not be distinguished from *Sulfitobacter pontiacus* (Pukall et al., 1999). Whether to consider other gene sequences, significant differences in the patterns of whole cell proteins, or restricted or amplified nucleic acids should be decided case by case.

The other examples refer to medically important organisms. Strains of *Escherichia coli* and *Shigella dysenteriae* are extremely closely related and exhibit DNA hybridization values as high as 89% (Brenner, 1973). Nevertheless, for epidemiological purposes the two taxa are not considered strains of the same species but are presently allocated to two different genera. On the other hand, certain strains of *Clostridium botulinum* are remotely related only by 16S rDNA analysis, they share less then 10% DNA-DNA similarity as measured by hybridization, and they are more closely related to other *Clostridium* species than they are related among themselves. However, as all of them express a botulinum toxin, which immediately guides the physician in the therapy of botulism, no attempts have been made to alter the classification of these strains (Fig. 2).

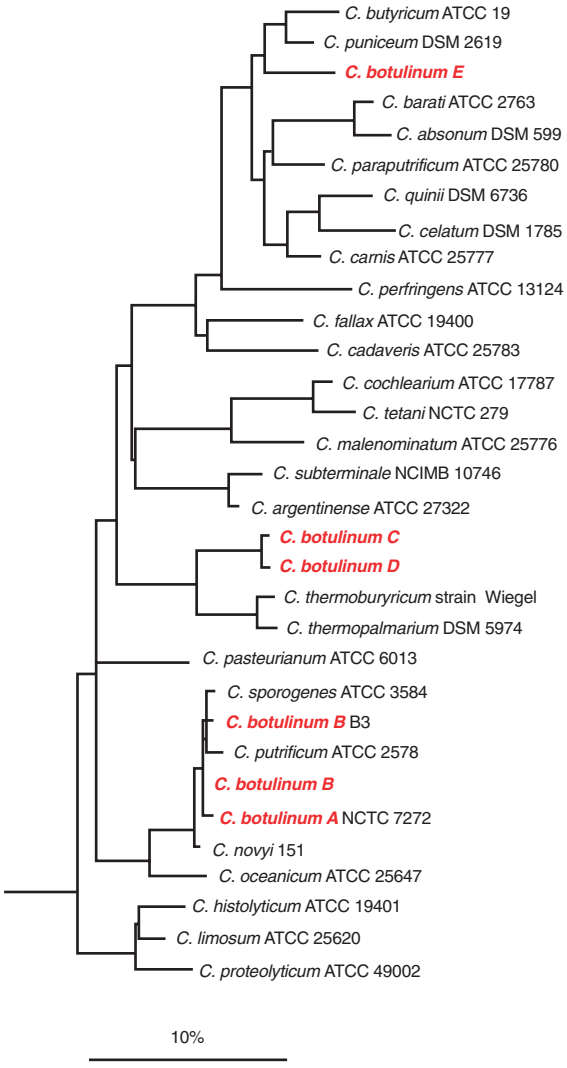

Fig. 2. The phylogenentic relatedness of *Clostridium botulinum* strains exhibiting different serotypes among strains of different *Clostridium* species of group I (Collins et al., 1994). The 16S rDNA dendrogram is a detail of the ARB tree. The scale bar corresponds to 10 nucleotide substitutions per 100 sequence positions.

1. Despite the recommended value of 70% DNA similarity, taxonomists working with some defined prokaryotic groups have altered this value to come to a better correlation between phenotypic and genotypic similarities. Within the family Pasteurellaceae, a DNA-DNA reassociation value of and above 85% describes a species (Mutters et al., 1985). Similar values have been found for the interspecies relatedness of *Blastomonas pertussis*, *Blastomonas parapertussisi* and *Blastomonas bronchiseptica* (Kloos et al., 1981) and between members of the spotted fever group of *Rickettsia* (Walker, 1989). Another well-known example in the literature is the fate of the members of the genus *Brucella* (Moreno, 1997). This genus contains six species (Meyer, 1990), in spite of the fact that DNA-DNA reassociation values separate these species above 98% similarity (Verger et al., 1985). For the workers in the field, the presence of individual DNA restriction patterns, phenotypic and antigenic properties, and, above all, the distinct biological behavior of the species, e.g., host range and pathogenicity, are more indicative than the strict application of the general rule of separating species.

2. In the case of 16S rDNA, if similarity values indicate an approximately equidistant relationship to members of different genera, the diagnostic properties given for these genera must be tested for the isolate. Such highly related genera have been described in the order Actinomycetales, α and γ subclasses of Proteobacteria, and the *Bacillus* line of descent. If the properties match those of one of the genera, the identification process will be restricted to members of this genus and one has to proceed as indicated under 1. If the analysis of the genus-

specific properties reveals no match with any of the genera, it is likely that the isolate represents yet another closely related genus of this genus cluster and the description of the new species and the new genus will go hand in hand. Well-known examples are the closely related species of the genus *Rhizobium*. Nevertheless, at this high level of 16S rDNA relationship, the analysis also will have to include the only technique recommended for the elucidation of intragenus relationships, i.e., the determination of DNA-DNA reassociation values between the species of the new genus and the species of the neighboring genera. Another example has been described for the genera *Blastomonas* and *Erythromonas* in the alpha subclass of Proteobacteria (Yurkov et al., 1997). The type strain of *E. ursincola* shares 99.2% 16S rDNA similarity with the type strain of *B. natatoria*. Based upon the presence of bacteriochlorophyll a in *E. ursincola* and its absence in *B. natatoria*, it was concluded that these organisms represent different genera. The DNA-DNA reassociation value of about 40% for these two strains (Tindall, pers. comm.) supported the taxonomic separateness of the two species. If, however, future studies show that the presence of bacteriochlorophyll a is not a unifying property of *E. ursincola* strains, or if bacteriochlorophyll is found in strains of *B. natatoria*, the taxonomic rationale for having two genera will diminish.

3. If the new isolate shares less than 97% sequence similarity with the nearest phylogenetic neighbor, then as many taxonomists now recommend (Stackebrandt and Goebel, 1994) DNA-DNA reassociation studies are unnecessary, because the latter values will range clearly below the 70% reassociation borderline value recommended for species definition. Indeed, a survey of articles of volume 48 of the *International Journal of Systematic Bacteriology* indicates that for about 30% of all newly described species sharing less than 97.5% 16S rDNA sequence similarity with their closest neighbor, DNA-DNA reassociation has not been determined. In those cases in which DNA-DNA reassociation values were provided, the values are clearly below the 70% threshold (Fig. 1).

Several genes other than the one coding for 16S rDNA have been sequenced recently to test the discriminating power of 16S rRNA genes. Interestingly, in *Shewanella* species, genes coding for gyrB were found to have a less conservative primary structure (Venkateswaran et al., 1998) than those coding for 16S rRNA and thus appear to be better suited for the elucidation of close relationships. In *Pseudomonas*, results of gyrB analysis matched those of 16S rDNA analysis, but only when the highly variable regions were omitted from the latter molecule (Yamamoto and Harayama, 1998; Yamamoto et al., 1999). Other genes used were the chaperonin GroRL (Viale et al., 1994), heat shock protein (hsp65) for strains of the *Mycobacterium avium* complex (Swanson et al., 1997), a gene (sodA) coding for a manganese-dependent superoxide dismutase in streptococci (Poyart et al., 1998), the ompA gene of Rickettsiae (Fournier et al., 1998), the mba gene fragments of *Ureaplasma* (Knox et al., 1998), or the RNAse P in Actinobacteria (Cho et al., 1998).

Increasingly, the spacer regions (ITS) separating the genes coding for the 16S rRNA and the 23S rRNA are used to determine inter- and intraspecies relatedness (Barry et al., 1991; Gü and Stanisich, 1996; Leblond-Bourget et al., 1996). Though this approach is currently used mainly for differentiation but not for the delineation of species, this and other molecular techniques such as chromosomal DNA fingerprinting (RFLP, restriction fragment length polymorphism; AP-PCR, arbitrarily primed PCR; ERIC-PCR, enterobacterial repetitive intergenic consensus PCR), gene fingerprinting (rDNA-RFLP analysis; Vaneechoutte, 1996), or ribotyping (Grimont and Grimont, 1986; Webster et al., 1994) can be useful in revealing the homo- or heterogeneity of strains of a species.

The Phenotypic Circumscription of a Species

In contrast to the rather stringent genomic definition of a "species," the phenotypic characterization of a new species is very variable. The properties to be investigated depend upon those indicated as being specific for the genus and on the set of characters already indicated for discriminating between species described for the genus.

Extensive morphological and ultrastructural characterization must be presented, especially for species of novel genera. Records on enrichment and isolation, motility, colony characterization, optimal growth conditions, growth requirement and substrates, and on base composition of DNA are parts of a set of characterizing features. Analysis of special features is required for certain taxa, such as antigenic characterization for *Leptospira* and mycoplasmas. Many of the properties to be provided for the description of a species are listed either in the descriptions of minimal standards, which are available for species of some genera, or they are compiled in *Bergey's Manual of Systematic Bacteriology*. Information about the phylogenetic position of a putative new type strain facilitates the selection

of such features and guides the search for taxonomically relevant properties.

Delineation of the Genus

The definition of a genus given by Cowan (1968) has not been changed by the input of molecular data. Cowan states that genus is "... one of the basic ranks in the hierarchical systems used in biology, and probably the highest rank with any significance in microbiology. In position between FAMILY and SPECIES, it is best considered as a collection of species with many characters in common; unfortunately no one has indicated the extent of this sharing of characters, and it is purely a matter of personal judgement ... as to what constitutes a genus. Like the SPECIES, the genus is a subjective concept without any foundation in fact."

A significant finding of the analysis of rRNA, rDNA and DNA-DNA reassociation studies was to point out the genetic heterogeneity of many phenotypically defined genera. Since genus and species are those ranks for which proper descriptions are needed most urgently, the new results were both confusing and encouraging (Stackebrandt and Woese, 1984): confusing in a sense in that in many cases, the working basis (the genus) had to be redefined after genera described on traditional grounds were found to be phylogenetically incoherent or after the type species was found to actually be a member of a different genus. Examples have been described for *Methanobacterium*, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Clostridium*, *Streptococcus*, *Flavobacterium*, *Bacteroides*, *Arthrobacter*, *Micrococcus*, *Brevibacterium*, *Nocardia* and several genera of phototrophic organisms, but it should be stressed that almost each genus was involved in the reclassification process to a varying degree. Some examples are listed in Table 4.

The results of the reclassification process, i.e. the elimination of misclassified strains, not only resulted in the description of genomically and phenotypically homogeneous genera, but also led to the reduction of species number per genus (Table 4).

A new genus has to be described when a strain or a strain cluster is shown to branch outside the radiation of a validly described genus and the isolated phylogenetic position is accompanied by distinct phenotypic properties not found among the neighboring genera. On the other hand, the placement of a new taxon with a novel pattern of phenetic properties within the radiation of a genus may point towards its taxonomic heterogeneity, which consequently may lead to the dissection of the genus. The decision about which phenotypic properties to use for the circumscription of a novel genus is up to the taxonomist but depends to some extent upon the description of the neighboring genera. The genus-specific characteristics must be present in each species of the genus. The following is a short list of examples of highly to moderately related genera and their discriminating properties:

Chemotaxonomic properties: In the order Actinomycetales for example the high degree of chemical diversity in the peptidoglycan, fatty acids, polar lipids, menaquinones, whole cell sugars or teichoic acid offers superb diversity at the epigenetic level to delineate genera. The correlation with phylogenetic analysis is so high that the finding of a new combination of such patterns indicates a new genus (Embley and Stackebrandt, 1994).

Morphological, chemotaxonomic and growth properties: The main basis for the division of the former genus *Bacillus* into eight genera has been the extensive phylogenetic analysis of its species (Stackebrandt et al., 1986; Ash et al., 1991; Rainey et al., 1993). The separation into several genera is based mainly upon the chemical structure of peptidoglycan, cell shape, spore shape, anaerobic growth, optimum pH, growth in 10% NaCl, and cellular fatty acids. As compared with the actinomycete genera, the importance of chemotaxonomic properties is low. The most species-rich genus, *Bacillus* (>60 species) itself, is heterogeneous with respect to amino acid composition of peptidoglycan, spore shape, anaerobic growth, presence of swollen sporangium and other features, and it can be expected that this genus will be subject of even further dissection.

Biochemical properties: The genus *Rhizobium* has been dissected into *Sinorhizobium*, *Azorhizobium*, and *Mezorhizobium* and these genera, together with *Allorhizobium*, *Mycoplana*, *Phyllobacterium*, *Agrobacterium*, *Bartonella bacilliformis* and *Blastobacter aggregatus* constitute a highly related group of mainly plant-associated bacteria (>92% 16S rDNA similarity). Phylogenetic analysis indicates that members of *Rhizobium* do not form a coherent genus but some members are more closely related to *Allorhizobium* (de Lajudie et al., 1998). The discriminative features between *Rhizobium* species and members of morphologically similar genera are predominantly results of carbon assimilation tests.

Morphological, physiological and growth properties: The number of genera within the family Chromatiaceae has recently been significantly enlarged, following the dissection of *Cromatium* into six genera and the reclassification of two *Thiocapsa* species as the type species of two novel genera (Imhoff et al., 1998b). This move was due to the phylogenetic heterogeneity of the genera involved though no novel phenotypic data were found that would clearly support the

Table 4. Some examples for the dissection of phenetically defined genera by transfer of species into phylogenetically coherent genera.

Phenetic definition	Number of species: Before	Number of species: Transferred reclassification	Emerging phylogenetically coherent genera	Higher order affiliation
Bacillus	124	36	*Bacillus*	
		3	*Alicyclobacillus*	
		19	*Paenibacillus*	
		10	*Brevibacillus*	Bacillaceae
		3	*Aneurinibacillus*	
		1	*Virgibacillus*	
Bacteroides	65	40	*Bacteroides*	Bacteroidaceae
		1	*Ruminobacter*	Succinivibrionaceae, γ-subclass of Proteobacteria
		7	*Porphyromonas*	Bacteroidaceae
		21	*Prevotella*	Bacteroidaceae
		1	*Anaerorhabdus*	Cytophagales
		1	*Megamonas*	not known
		1	*Rikenella*	Cytophagales
		1	*Mitsuokella*	Sporomusa subbranch of Clostridium group
		1	*Dichelobacter*	γ-subclass of Proteobacteria
		1	*Fibrobacter*	Fibrobacter line of descent
		1	*Sebaldella*	Fusobacterium line of descent
		1	*Dialister*	Sporomusa subbranch of Clostridium group
		1	*Campylobacter*	ε-subclass of Proteobacteria
		1	*Capnocytophaga*	Cytophagales
		1	*Tissierella*	Clostridium group
Brevibacterium	26	16	*Brevibacterium*	Brevibacteriaceae, Actinomycetales
		4	*Curtobacterium*	Microbacteriaceae, Actinomycetales
		3	*Corynebacterium*	Corynebacteriaceae, Actinomycetales
		2	*Cellulomonas*	Cellulomonadaceae, Actinomycetales
		4	*Microbacterium*	Microbacteriaceae, Actinomycetales
		1	*Arthrobacter*	Micrococcaceae, Actinomycetales
		1	*Desemzia*	Camobacterium group, Enterococcaceae
		1	*Exiguobacterium*	Bacillaceae
Clostridium	143	14	*Clostridium*	Clostridium subline of Gram-positive bacteria, Clostridiaceae
		1	*Oxalophagus*	Bacillaceae
		1	*Paenibacillus*	Bacillaceae
		1	*Eubacterium*	Clostridiaceae
		1	*Syntrophospora*	Clostridiaceae
		1	*Oxobacter*	Clostridiaceae
		2	*Moorella*	Clostridiaceae
		2	*Thermoanaerobacter*	Clostridiaceae
		2	*Thermoanaerobacterium*	Bacillaceae
		1	*Caloramator*	Bacillaceae
		1	*Filifactor*	Clostridiaceae
		1	*Sporohalobacter*	Haloanaerobiales
Flavobacterium	40	21	*Flavobacterium*	Flavobacteriaceae
		1	*Empedobacter*	Flavobacteriaceae
		2	*Sphingomonas*	Zymononas-group, α-subclass of Proteobacteria
		2	*Microbacterium*	Microbacteriaceae, Actinomycetales
		1	*Halomonas*	Halomonadaceae, γ-subclass of Proteobacteria
		1	*Myroides*	Flavobacteriaceae
		1	*Planococcus*	Bacillaceae
		1	*Cytophaga*	Cytophagales
		1	*Vogesella*	β-subclass of Proteobacteria
		1	*Telluria*	β-subclass of Proteobacteria
		1	*Marinobacter*	γ-subclass of Proteobacteria

reclassification process. Consequently, the traditional taxonomic markers (motility, presence of gas vesicles, morphology, salinity and temperature optimum, vitamin requirement, base composition of DNA, and chemoautotrophy) were reassessed and new patterns of differential characteristics were proposed to describe the 14 genera of Chromatiaceae.

End products of carbohydrate fermentation, morphology and chemotaxonomy: The genus *Bacteroides* has been a dumping ground for many phylogenetically misclassified strains, the extent of which was only unraveled by 16S rDNA analysis. As Table 3 shows, 12 new genera have been established for former *Bacteroides* species, some of which are related to members of the Proteobacteria.

The availability of a most comprehensive phylogenetic framework, covering the majority of described species, leads to the recognition of genus boundaries and, consequently, to the establishment of phylogenetically homogeneous genera. Genera in which species are described following their phylogenetic analyses are also mostly homogeneous, e.g. genera *Campylobacter*, *Helicobacter* and *Arcobacter* (Fig. 3). Nevertheless, in some areas of the phylogenetic tree the reclassification process did not parallel the progress in the recognition of relatedness. The prime examples are the deeply branching lineages of the *Clostridium-Bacillus* subline of Gram-positive bacteria, e.g., *Clostridium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, and species of other genera that are phylogenetically heterogeneous. Another example is the family Pasteurellaceae, in which members of the genera *Pasteurella*, *Haemophilus* and *Actinobacillus* are not yet reclassified to match their phylogenetic relatedness (Fig. 4). The phylogenetic branching clearly indicates the inappropriateness of some classical taxonomic properties to define genera, but novel discriminating characteristics to circumscribe the new emerging genera have not yet been found. The features identified as being of little taxonomic value are the combination of: 1) Gram-positive staining, rod-shaped morphology, spore formation, anaerobic metabolism lacking sulfur reduction (*Clostridium*); and 2) Gram-positive staining behavior, morphology, lack of spore formation and anaerobic metabolism (*Peptococcus*, *Ruminococcus*, *Eubacterium*). The situation is complicated even more by the branching of the spherical, spore-forming *Sarcina* species within the radiation of the

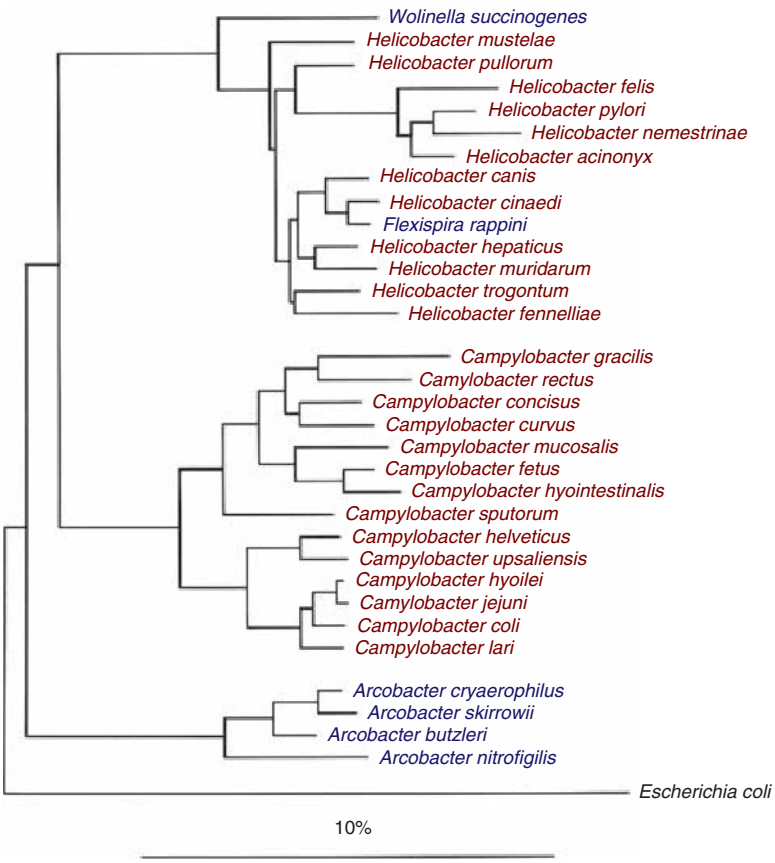

Fig. 3. 16S rDNA dendrogram of the *Campylobacter-Helicobacter-Arcobacter* line of descent, classified as the ε-subclass of Proteobacteria. The intrageneric structure of the genera is taxonomically coherent. The scale bar corresponds to 10 nucleotide substitutions per 100 sequence positions. The dendrogram was generated by the neighbor-joining algorithm (Felsenstein, 1993).

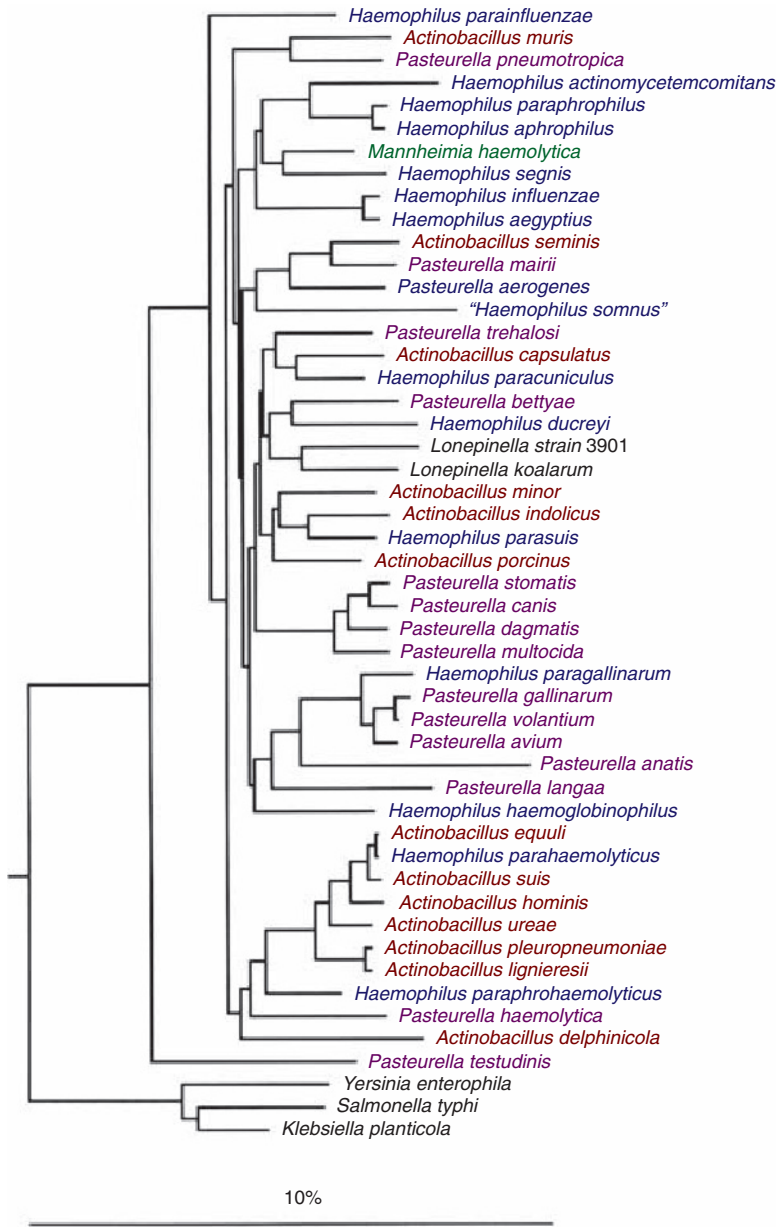

Fig. 4. 16S rDNA dendrogram of the Pasteurellaceae, a member of the γ-subclass of Proteobacteria. Due to the lack of discriminating phenotypic characteristics the intrageneric structure of the genera is taxonomically very incoherent. The scale bar corresponds to 10 nucleotide substitutions per 100 sequence positions. The dendrogram was generated by the neighbor-joining algorithm (Felsenstein, 1993).

Clostridium cluster I (according to Collins et al., 1994) that contains the type species and through the intermixing of Gram-negative species (Cluster IX) with Gram-positive *Clostridium*-type organisms (Schleifer et al., 1990; Stackebrandt and Rainey, 1997). As most of the genera defined by Gram-negative species are phylogenetically coherent taxa, such as *Sporomusa*, *Selenomonas* and *Pectinatus*, the misclassified non-type species of *Clostridium*, *Eubacterium* and some other genera must be reclassified, provided phenotypic properties are available that would support the reclassification. This information, however, is missing for most of the lineages prone to reclassification, except for a few examples in which new genera have been described, e.g., *Moorella*, *Filifactor*, *Thermoanaerobacter* and *Thermoanaerobium*.

Genera May Have Different Phylogenetic Depth

Individual genera that are phylogenetically coherent and have been properly described phenotypically may vary significantly in their phylogenetic depth, i.e., the relative time that passed after the two most unrelated members of a genus separated from a common ancestor. It has been noted (Stackebrandt, 1992) that in contrast to phylogenetically defined taxa, those based on

phenotypic description in the past have no depth per se, as nonmolecular properties are not able to measure relative evolutionary time. Genera can either have few or many species, but as long as secondary or tertiary semantides are analyzed, nothing can be said about when the common ancestor of the respective taxon evolved. The definition of a genus assumes that the phenotypic properties of its species are uniformly distributed among the species while the underlying structure of those genes used for phylogenetic analysis (and probably of other genes as well) may vary dramatically. Actually, for 16S rRNA genes, the degree of sequence variation between members of a genus may range from small (for instance, above 97% for *Micrococcus*), to moderate (92% for *Streptococcus*), to significant (about 79% for *Spirochaeta*) (97, 92, and 79% are all sequence similarity values.) Except for some recently evolved eukaryote-associated symbionts, it is still impossible to correlate sequence divergence of a given gene with a time scale, but it is possible to determine the relative age of a taxon from a comparison of 16S rDNA similarity values of the most unrelated species of a genus. For practical reasons, three categories named "age groups" have been described (Stackebrandt, 1988). These "groups" are hypothetical entities not clearly delineated in the hierarchical tree.

Members of the first "age group" were assumed to have evolved during the anaerobic phase of evolution. Species of such genera are in most cases well separated, which explains why DNA-DNA hybridization fails to relate them. Representative genera are found in the several families of methanogenic bacteria, as well as in *Bacteroides*, *Spirochaeta*, and *Clostridium* (senso strictu, i.e., cluster I) and their several phylogenetically related lineages, which need to be reclassified or have already been reclassified. The subjective selection of genus-specific phenotypic traits has not permitted determination of the actual phylogenetic depth of these taxa. It is of interest to note that certain phenotypic traits of taxonomic significance, including morphology, spore formation, proteins of the photosynthetic apparatus, and biochemical properties, appear to have remained constant over billions of years. The dramatic differences between the molecular and the phenotypic level appear to be rooted in mechanisms that cause a disjunction between the evolution of genotype and phenotype. This problem has been discussed by Nanney (1984), who suggested that the highly conserved morphological characteristics of eukaryotic species are the result of a compounding of molecular properties that may themselves be very divergent. As a consequence, divergent structure-forming components may interact in a way that conserves the resulting morphological structure, as well as biochemical pathways and physiological properties, in the case of prokaryotes.

Members of the second "age group" are those genera whose ancestors evolved during the transition period when the earth passed from an anaerobic to an aerobic environment. Descendants of this group are either facultatively anaerobic or aerobic. Most species are moderately related, but groups of closely related species exist. Their existence may be viewed as a more recent speciation event, causing strains of certain species to evolve faster than others. The reasons for this are not known, but changes in the evolutionary rate caused by changes in the environment may play a dominant role. Examples are found in *Bacillus*, *Streptococcus*, *Lactobacillus*, and *Corynebacterium*.

Members of genera belonging to the third "age group" probably evolved during the aerobic phase of evolution. Genera are very shallow phylogenetic taxa, since even the most distant species are still highly related. Most of these genera can be separated easily from closely related taxa by a combination of chemotaxonomic markers. The presence of different phenotypes in closely related genera is an indication of rapid evolution at the overall DNA level. This is seen not only in many actinomycete genera, e.g., *Streptomyces*, *Actinomadura*, *Staphylococcus*, *Listeria*, but also in many Gram-negative genera, e.g., *Vibrio*, *Shewanella*, *Rhizobium*, *Hyphomicrobium* and most genera of the Enterobacteriaceae. Genes coding for ribosomal RNA, on the other hand, are so conserved in their primary structure that changes at the level of the overall chromosome do not manifest within a short period. It is therefore obvious that DNA-DNA reassociation studies will reflect the actual relatedness between the species most closely, whereas comparison of complete rDNA sequences often fails to reliably determine the intrageneric structure of these taxa.

The presence of groups with varying phylogenetic depth explains why initial attempts have failed to delineate taxa by a purely phylogenetic—and inflexible—approach. The following example demonstrates this impracticability. For the methanogenic bacteria (Balch et al., 1979), a lower range of S_{AB} values of 0.55 to 0.65 was set for species differentiation (S_{AB} values are now replaced by rDNA similarity values, and S_{AB} values of 0.55 to 0.65 correlate with about 88 to 91 % similarities). If this same range were applied to the bacterial genus *Staphylococcus*, all of its members would have to be reduced to a single species. Application of the phylogenetic genus definition to the methanogens on the branching pattern of the order actinomycetes would have

even more dramatic consequences in that all genera would be united in a single genus (Fox and Stackebrandt, 1987). Using operational definitions as the sole criteria for taxon delineation, most of the existing phenotypically well defined and phylogenetically coherent genera would have to be considered invalid. Individual genera would have to be dissected to form several new genera with identical properties (that were once used to combine its members), or different genera, whose members exhibit such a wide variety of phenotypes that the classification would be of little practical value, would have to be united. The effects of the different delineation strategies on the classification of actinomycetes have been demonstrated schematically (Fox and Stackebrandt, 1987).

It must, however, be remembered that the delineation of a genus in bacteriology does not have to follow the historical record at all but depends upon the availability of phenotypic data coupled with the opinion of the taxonomists (Cowan, 1968). The availability of a phylogenetic framework has initiated a trend that led to the description of genera as phylogenetically shallower than genera based solely upon phenotypic properties. This is most dramatically seen in the dissection of some former species-rich genera, e.g., *Micrococcus*, *Brevibacterium*, *Pseudomonas*, and *Bacteroides* that has led to the description of many monospecific genera.

Classification Is a Dynamic Process (Stackebrandt, 1991)

Microbiologists are aware that the available phylogenetic branching patterns, although sensational and revolutionary because of their new potential, only very incompletely reflect the actual situation in nature. Phylogenetic reconstructions are based on inferred homologies but, unless witnessed by the evolutionary history of taxa, i.e., by fossil data, cannot be considered definitive (Rothschild et al., 1986). Furthermore, the tree mirrors the presence of certain categories, e.g., the spirochaetes, the planctomycetes, and the thermotogas, that may be self-defining since they are emerging constantly, no matter which molecule and method are used. However, the isolated position of these groups, well defined by genotype and phenotype today, may disappear tomorrow when more organisms are investigated. Thus, whenever new information—either within established taxa or in neighboring groups—requires corrections, flexibility is called for and changes have to be made for the benefit of a better agreement between phylogeny and taxonomy. The main advantages of the phylogenetic system lie in its stability: only the rank (either vertical or horizontal) but not its place within the hierarchical structure will be changed—as happened in past systems (a comparison of *Bergey's Manual of Determinative Bacteriology* from the first through eighth edition is instructive).

Even the most convincing tree is always in a dynamic state; this forces taxonomists to stay flexible to adjust not only established ranks but also nomenclature according to new insights. One problem still remains: the original advantage of the tree—its objectivity (in so far as is possible)—is weakened by subjective (variable emphasis on characters) clustering of organisms. As in previous decades, the most practicable system (or parts thereof) will succeed against competing systems with less persuasive arguments. The ultimate goal is to establish a hierarchical system where all taxa show phylogenetic coherency and, at least for ranks below the family level, a great deal of phenotypic coherency as well. On the other hand, sufficient differences need to be known to distinguish taxa from each other by stable and easily determined characters. While phylogenetic coherency is easy to define, the term "phenotypic coherency" varies according to the taxonomist. Again, practical considerations must come before petty splitting or lumping.

Profound knowledge about the phylogenetic clustering of members of the taxa in search is prerequisite. An optimal survey would work with coded, unnamed organisms to judge the resulting branching pattern without prejudice. The study should include the type strain of the type species and, if any information is already available, the type strain of the most unrelated representatives of the taxon. Depending on numbers of strains investigated, degree of relationships, and cost effectiveness, studies will include DNA-DNA pairing. The resulting pattern depicts the relative branching order, that, depending on the size of the database and the selection of reference organism, will immediately yield information about the phylogenetic homogeneity of a group of isolates. In the second step, the branching pattern is superimposed with phenotypic data to delineate clusters of organisms which are phylogenetically coherent and easy to recognize by phenotypic characters. This is prerequisite not only for identification, but also to decide which of the several possible branching patterns best reflects phylogeny most closely. It should be mentioned in this context that in the presence of varying evolutionary rates, species with the greatest nucleic acid sequence similarity are not necessarily the most closely related; while programs that optimize branch length take care of this problem, numerical phenetic analyses in fact

would cluster these species as neighbors. The combination of taxon-describing characters will not be predictable in most cases and the search will have to be extended to features not previously considered of taxonomic significance. Still many phylogenetically coherent taxa exist for which appropriate characters have not been found as yet.

The Higher Taxa

Analysis of sequences of rDNA and genes coding for proteins are routine some 10 years after the introduction of the PCR technology and the automated sequencing process. Large-scale genome sequencing projects are underway and the availability of an enormous number of sequences of homologous genes will one day allow a much more precise measurement of the branching order of lineages. However, with new lineages emerging from pure culture studies and analysis of complex microbial communities, the main topic of discussion is presently not the definition of higher taxa but very early evolutionary events, such as those that led to the formation of the eukaryotic cell, and the data discussed are sequence analyses of genes such as hsp70 (chaperone protein), glutamate dehydrogenase, glutamine synthase, aspartate aminotransferase and others (Gupta and Golding, 1993; Gogarten et al., 1989). Lateral gene transfer effects must not be neglected when phylogenetic trees based upon heat shock proteins (hsp70) are interpreted (Gribaldo et al., 1999; Philippe et al., 1999). The question whether or not the Gram-positive bacteria are more closely related to the Archaea than to the Gram-negative bacteria (Gupta and Golding, 1996) is a problem that can not be resolved on the basis of a few signature stretches of the gene and the amino acid sequence derived therefrom. The problem awaits a better understanding of the fate of the molecule in early evolution and greater ability to interpret the sequence data from a single gene in context of the overall biology of the organism.

According to Woese and colleagues (Woese et al., 1990), extant organisms are grouped within one of three major lines of descent, for which the domain has been proposed. The term "taxon domain" has replaced the term "primary kingdom" (Woese and Fox, 1977) originally given for the three main lines of descent which, based upon 16S rRNA analyses, are as unrelated to each other as each of them is related to the eukaryotic line. With the higher resolution provided by complete sequences of small-subunit rRNAs and other homologous marker molecules, this picture has been confirmed. The archaeal lineage is clearly separated from the other prokaryotic lineage, the Bacteria, forming a sister group of the Eucarya.

The main issue discussed at the end of the 1970s was whether the tripartition of extant species is accompanied by phenotypic properties that would support the phylogenetic finding. These properties were believed to exist because molecular analyses revealed the presence of evolutionary ancient groupings. While characters shared between members of two of the three domains are of no use for placing strains in the phylogenetically correct kingdom, certain characters are indeed exclusive for a particular primary domain and hence of diagnostic value. However, one can not exclude the possibility that nonhomologous phenotypes occur among members of different domains that mirror common evolutionary origin. On the other hand, homologous properties may actually be found exclusively among members of two domains, but this distribution is not due to common ancestry but to horizontal gene transfer.

Besides the structure and nucleotide composition of the 16S rRNA, several epigenetic properties were unique to Archaea and supported the validity of the description of this domain. Above all they were the lack of a typical peptidoglycan (replaced by a pseudomurein, a proteinaceous wall, or a heteropolysaccharide; Kandler, 1982), the presence of ether-linked isoprene units, and the complex modification pattern of ribosomal RNAs. In addition, certain archaeal taxa exhibit unique properties, which are absent in other taxa of this domain and in any bacterial or eukaryal taxon. The most well recognized ones are the coenzymes involved in methanogenesis; the energy-generating bacteriorhodopsin, halorhodopsin, and other sensory rhodopsins contained in the purple membranes of halophiles; survival under hyperthermophilic conditions; and singular physiological features such as the presence of a modified Entner-Doudoroff pathway. Other features will be discovered through the comparative analyses of fully sequenced archaeal genomes.

Since publication of the last edition of the Prokaryotes (Balows et al., 1991), new main lines of descent have been shown to exist in the domains Archaea and Bacteria, and most lineages have been significantly extended by studies on pure cultures and analysis of environmental samples. Ranks above the genus level have been described for several of these main lineages and for some of their sub-branches. What has been stated about the unreliability of standardized sequence similarity values of a single molecule to define a genus is also true for all higher ranks from family to kingdom. Consequently, a coherent hierarchical system of prokaryote taxa does not exist. While for one lineage a fully hierarchical

structure has been provided (Stackebrandt et al., 1997), there is no consensus about the level of the highest rank of a lineage. Lineages that are approximately equivalent in phylogenetic depth (i.e., the 16S rDNA similarity value that separates the most remotely related members of that lineage) are called kingdom (within the domain Archaea), phylum (some of the lineages originally defined by Woese et al., 1985, e.g., *Chloroflexus*, *Chlorobium*, *Deinococcus*, *Thermus*, *Bacteroides*, *Clostridium-Bacillus*), class (Actinobacteria, Proteobacteria), or order (Aquificales, Thermotogales, Spirochaetales, Verrucomicrobiales, Chlamydiales, Planctomycetales).

The phylogenetically defined higher taxa stand side by side with higher taxa established in the pre-phylogeny era. Some taxa were found to match the phylogenetic circumscription, e.g., the class Mollicutes, the orders Spirochaetales, Chlamydiales, and Myxobacteriales and the family Enterobacteriaceae, while others had to be emended or redefined on the basis of 16S rDNA sequence data to fit into a phylogeny-based hierarchical system, e.g., Micrococcaceae and Pseudomonadaceae. Yet other higher taxa such as the Bacillales and Clostridiales are still awaiting a formal revision, as phylogenetic evidence strongly points towards their dissection.

The Archaea

Concerning the phylogeny-based description of higher taxa, the domain Archaea has received considerable attention, primarily because of research activity in the laboratories of Ralph Wolfe and Carl Woese at the University of Illinois at Urbana-Champaign. Shortly after archaebacteria (as these organisms were named in 1979) were recognized, Balch et al. (1979) provided a comprehensive hierarchical system based on the assumption that the rate of 16S rRNA evolution in these organisms was similar. Consequently, species, genera, families and orders were delineated by ranges of S_{AB} values of 16S rRNA,which were 0.55–0.65, 0.46–0.51, 0.34–0.36 and 0.22–0.28, respectively. Later it became obvious that different bacterial species not only evolve a different mode and at a different rate (Woese et al., 1985c), but also their rRNA and rDNA may have different G+C content (Woese et al., 1991; Rainey et al., 1993; Liesack et al., 1992). The result was artificial misplacement of bacterial species in the phylogenetic dendrogram. The suggestion not to use a rather inflexible range of phylogenetic distances for the delineation of any taxon (Fox and Stackebrandt, 1987; Stackebrandt, 1992) has been accepted and applied to more recent descriptions of higher taxa in the domain (Burggraf et al., 1997).

When higher taxa were proposed for the second archaeal kingdom, the Crenarchaeota, only a few species had been described. It is therefore not surprising that the phenetic descriptions of the orders Thermoproteales (Zillig et al., 1981), Sulfolobales (Stetter, 1989), as well as those of the families Thermoproteaceae (Zillig et al., 1981) and Desulfurococcaceae (Zillig et al., 1982), Pyrodictiaceae and Thermofilaceae (Burggraf et al., 1997), are virtually the same as the genera they encompass. The inclusion of more organisms in a higher taxon, e.g., Thermoproteales and Thermoproteaceae (Burggraf et al., 1997) broadens the phenetic description.

The Bacteria

A similar situation is encountered among the deeply branching lineages of the domain Bacteria. Encouraged by the ease at which higher taxa were described for archaeal lineages, orders and families were almost simultaneously described with the recognition of a new genus or a cluster of neighboring genera. Examples are the order Aquificales, embracing the family Aquifexaceae, and the genera *Aquifex*, *Calderobacterium*, *Hydrogenobacter*, and *Thermocrinis*, as well as the order Thermotogales, embracing the family Thermotogaceae and several genera, e.g., *Thermotoga*, *Geotoga* and *Petrotoga*.

These examples demonstrate that the description of a higher taxon for a phylogenetic lineage is facilitated by the small number of organisms and by the isolated position of the lineages. The situation is different in the four main bacterial lineages, which show a complex phylogenetic structure: the Gram-positive bacteria (division Firmicutes), the class Proteobacteria, the Bacteroidaceae-Cytophagales line of descent, and the cyanobacteria phylum. Within the cyanobacterial lineage, higher taxa have been described on the basis of morphology and, within the Prochlorophytales, of pigment composition. Analysis of 16S rDNA clearly demonstrates that the present affiliation of genera into the orders Chroococcales, Nostocales, Oscillatoriales, Pleurocapsales and Stigonematales is not always supported from a phylogenetic point of view.

One of the most unexpected relationships that emerged from the analyses of rRNA, rDNA, and certain genes coding for proteins was the specific grouping of the *Bacteroides* and *Cytophaga-Flavobacterium* lines of descent. No higher taxon has been proposed as yet for the lineage comprising the phylogenetic assemblage of genera but, with the exception of the phylogenetically coherent family Sphingobacteriaceae (Steyn et al.,

1998), organisms are related to the traditional higher taxa Cytophagales and Bacteroidaceae.

The Higher Taxa of the Gram-Positive Bacteria

The Gram-positive bacteria constitute the division Firmicutes (Gibbons and Murray, 1978), which, with the exception of members of *Deinococcus*, appears to form a phylogenetically coherent taxon. The common ancestry of the two major sublines, however, one embracing the actinomycetes, the other containing the clostridia, bacilli and their relatives, has not been convincingly demonstrated by rDNA analyses. If, for the time being, it is assumed that these organisms indeed share a common ancestry, then the deep separation between organisms with a DNA base composition of less than about 50 mol% G+C (the *Clostridium-Bacillus* lineage) and those with a higher G+C content (the Actinobacteria) may facilitate resolution of their hierarchical structure.

A hierarchical classification system has been described for the actinomycetes and their relatives (Stackebrandt et al., 1997). The rationale for doing so was based on the fact that nearly all type strains of validly described species were characterized phylogenetically and that the genera constituted phylogenetically and phenetically coherent taxa. The decision to classify phylogenetically neighboring genera into families, neighboring families into suborders, and to continue up to the level of the class Actinobacteria was done irrespective of phenetic properties on which higher classification of these genera was based in the past. Rather than focusing on characteristics such as morphological, physiological and chemotaxonomic traits, which, except for the presence of mycolic acids, have no or only restricted phylogenetic meaning above the genus level, ranks were defined on the basis of emerging phylogenetic clusters and the presence of taxon-specific rDNA signature nucleotides. As a consequence the actinomycete proper has been classified into 5 subclasses, 6 orders, 10 suborders, and 35 families. Each taxon is characterized by a set of 16S rDNA signature nucleotides which were present in all or at least in the vast majority of members of a taxon at the time of its description. This way of circumscribing taxa is similar to the traditional, phenotype-based classification in that new members may have properties that differ to some extent from those of other members of the taxon. Consequently, the deviation may lead to an emendation of the taxon or, where the deviation is significant, to a dissection of the taxon. Any deviation from the signature pattern of a new member of a taxon may cause the taxonomist to revise the description of this taxon. In contrast to the past, lumping or splitting of taxa will not dramatically change the classification system, although the transfer of a taxon into the neighboring taxon of the same level may occur in those cases where the branch points of taxa in the phylogenetic tree are not well resolved. Within the second major lineage of the Firmicutes, the situation is more complicated. This phylogenetic cluster contains several higher taxa described by phenetic characteristics, e.g., the orders Bacillales and Clostridiales as well as the families Bacillaceae, Clostridiaceae, and Peptococcaceae, but the phylogenetic structure of such taxa does not correlate at all with the phylogenetic suprageneric classification. The situation is even more complicated by the present inability to define a genus *Clostridium*, needed to start a comprehensive phylogenetic classification process. The only higher taxon phylogenetically described so far within the *Clostridium* lineage is the order Haloanaerobiales, containing the families Haloanaerobiaceae (Oren et al., 1984) and Halobacteroidaceae containing Gram-negative, fermentative, halophilic and anaerobic bacteria (Rainey et al., 1995). The phenetic order Bacillales, developed from an ancestor of the Clostridiales, embraces the phylogenetically and phenetically coherent class Mycoplasmatales and the families Streptococcaceae and Lactobacillaceae, next to other higher taxa to be described. It is obvious that any attempt to create a fully comprehensive phylogeny-based hierarchical classification system for the *Clostridium-Bacillus* lineage has to await a thorough reclassification of the majority of genera contained in the lineage. This process has started with the dissection of the genus *Bacillus* and the establishment of new genera but will continue at a slower pace than in other lineages because of the significant degree of discord between phenetic classification and the phylogenetic position of species.

The Higher Taxa of the Proteobacteria

Members of the Proteobacteria are distributed into five subclasses. From the beginning of the molecular era of taxonomy, many of the Gram-negative taxa were investigated in parallel by 16S rRNA cataloging and determination of 16S rDNA similarities (Fox et al., 1977; Palleroni et al., 1973), and the agreement of the branching patterns was convincing. Almost complete 16S rDNA sequences have been generated for these and other organisms now seen to be members of the Proteobacteria. The phylogenetic clustering of organisms of quite different phenotypes initially surprised traditional microbiologists and gave the first indication that conventional classification does not reflect natural relationships.

Phototrophic bacteria were found to be neighbors of nitrite-oxidizing and carbon-monoxide-oxidizing forms, and organisms associated with eukaryotic cells were more closely related than originally believed (e.g., *Agrobacterium*, *Rhizobium*, *Brucella*, *Rochalimea*). But most unexpectedly, almost all of the more general characters used so far in classification lost their significance as taxon-describing features, e.g., photosynthesis (Stackebrandt et al., 1988a), carbon monoxide oxidation (Auling et al., 1988), methane- and methanol oxidation (Bowman et al., 1993), as well as all kinds of cell shapes, such as helical (Woese et al., 1982), budding (Stackebrandt et al., 1988b), or prosthecate (Schlesner et al., 1989). Stimulated by the phylogenetic data, supporting evidence has subsequently become available from chemotaxonomic studies. Among these criteria are polyamine patterns (Busse and Auling, 1988), ubiquinone types (Urakami et al., 1989), fatty acid compositions (Urakami and Komagata, 1987; Sittig and Schlesner, 1993), chemical compositions of lipid A, and the core region of lipopolysaccharides (Weckesser and Mayer, 1988).

The process of transforming phylogenetic evidence into the description of higher taxa is slow. This is not only true for a formal description of the subclasses but especially for the groupings within the subclasses. The reasons are obvious: 1) emphasis is placed on the generation of phylogenetically coherent genera; 2) neighboring genera may differ from each other significantly in phenotypic properties, which excludes the provision of phenetically coherent higher taxa; 3) the phylogenetic distances separating groups of genera from each other are small and the order is most likely distorted by new sequence entries; and 4) many new proteobacterial genera are presently described that may result in emendations and changes in the order of higher taxa.

The richest family structure is present in the γ-subclass in which a large number of genera, which are not necessarily phylogenetically coherent (e.g., *Pasteurella*, *Haemophilus*, *Serratia*, *Enterobacter*), are members of phylogenetically coherent families such as *Pasteurellaceae* and *Enterobacteriaceae*. Other well-defined families are Vibrionaceae, Aeromonadaceae, Legionellaceae and other families shown in Fig. 5. The only family that seems to be phylogenetically incoherent is Methylococcaceae. While the majority of genera of this family are positioned as shown in Fig. 4, the genus *Methylococcus* branches adjacent to the family Chromatiaceae. The other subclasses are less formally structured. Among the α-subclass, the Acetobacterceae, Rhizobiaceae, and the Rickettsiales constitute phylogenetically rather homogeneous taxa, while in the β-subclass, which appears as a

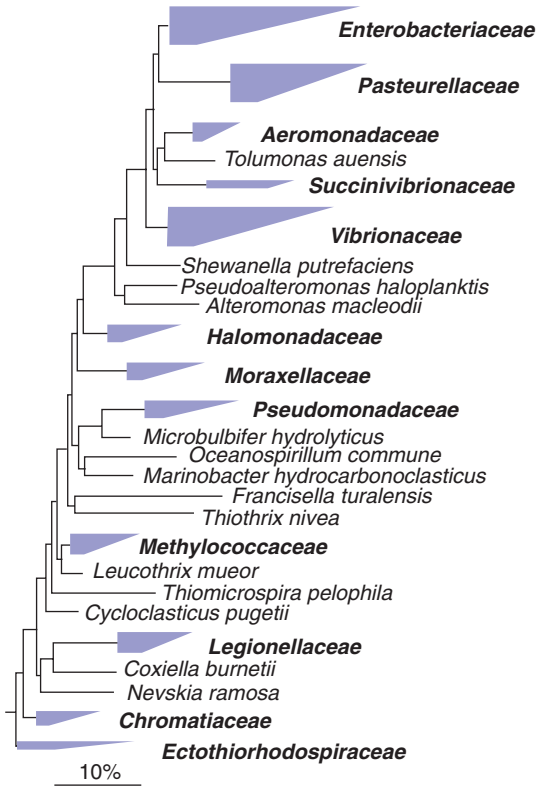

Fig. 5. 16S rDNA dendrogram of the γ-subclass of Proteobacteria, showing the position of phylogenetically coherent families. The 16S rDNA dendrogram is a detail of the (ARB tree). The scale bar corresponds to 10 nucleotide substitutions per 100 sequence positions.

subgroup of the γ-subclass, this refers to *Comamonadaceae* and *Neisseriaceae*. Within the δ-subclass the order *Myxobacterales* provides one of the rare examples in bacteriology in which the taxonomic structure as derived from phenotypic characterization, i.e., complex and highly ordered morphologic processes, is indeed valid indication of phylogenetic structure (Spröer et al., 1999).

Application of the Polyphasic Approach to Classification

The following is an example of the polyphasic approach to systematics (Fig. 6). First, it is assumed that the organisms abbreviated A through H have no taxonomic history but present novel isolates. In reality this is not the case because they represent well-known actinomycete genera and species (Stackebrandt et al., 1997). Analysis of 16S rDNA data leads to the placement of their sequences within the radiation of members of the class Actinobacteria, forming a sister-branch of the family Pseudonocardiaceae. As the phylogenetic depth of the new

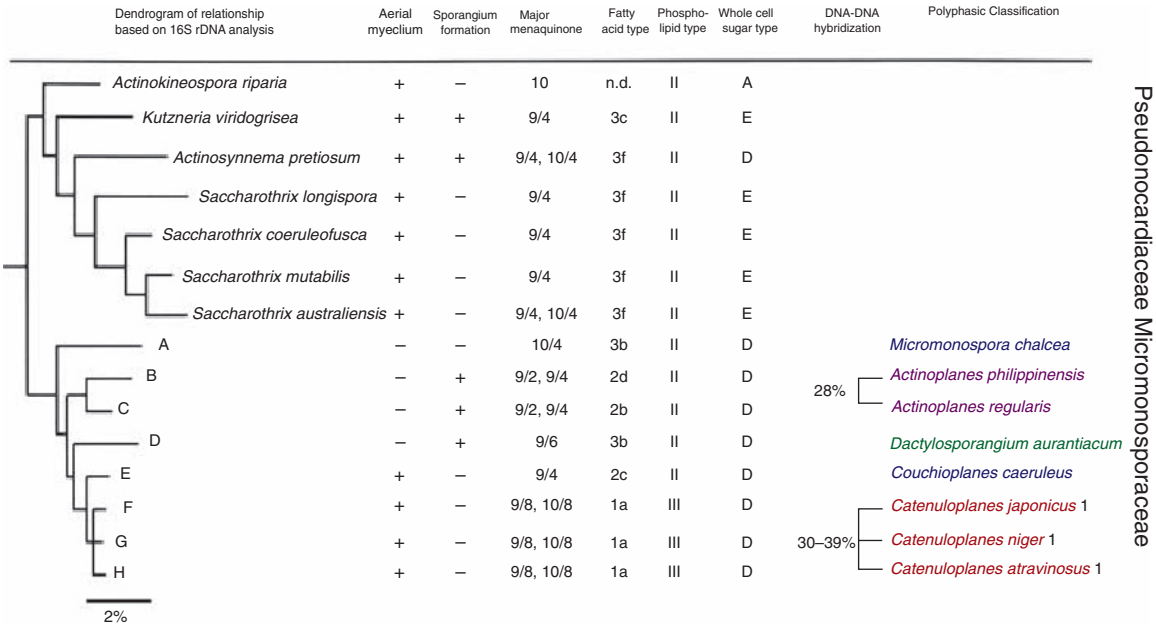

Fig. 6. Example of a polyphasic approach to bacterial taxonomy. The present classification is based on phylogenetic coherence of genera delineated from each other on the basis of morphological and chemotaxonomic properties. The selection of suitable properties depends upon the taxon under investigation. For clarity, the information on fatty acids, phospholipids, and whole cell sugars is abbreviated (*Bergey's Manual of Systematic Bacteriology*, Wilkins & Wiley, Baltimore, 1986). [1], data from Tamura et al., 1993, 1995.

lineages is comparable to that of the genus *Saccharothrix*, lineages A through H could constitute a novel genus. However, as the rate of evolution is different in members of different genera, the newly emerged phylogenetic cluster might well embrace two or more genera. Hence, the provision of a phylogenetic dendrogram alone does not a priori permit conclusions about the rank of taxa. According to the polyphasic approach, one would try to allocate as many genetically stable characters as possible to the isolates A to H to find characters that are unique to one phylogenetic cluster but different from neighboring clusters. As some characters may be shared by different genera, it is the presence of a unique pattern of characters that decides if a rank is delineated from its neighbors. Figure 6 lists some of the morphological and chemotaxonomic characters used in the polyphasic classification of actinomycetes. As derived from the example of the Pseudonocardiaceae, each genus is characterized by a unique set of morphological and chemical properties. Species of the genus *Saccharothrix* share the genus-specific pattern (but they can be distinguished from each other by physiological reactions). Patterns obtained for the (hypothetically) new organisms are novel for the actinomycetes genera and indicate five subgroups worthy of genus rank. To determine whether the isolates with identical phenetic patterns constitute individual species, DNA-DNA reassociation experiments must be performed. Isolates B and C (Stackebrandt et al., 1983), as well as isolates F, G and H (Yokota et al., 1993) represent individual species, as in each case the reassociation values are below 70%. Consequently the isolates can be classified into five new genera containing 8 new species which can be phenotypically separated (not shown).

The affiliation of the new monophyletic genera to a family on the basis of phenotypic data is not appropriate because of the great morphological and chemical diversity that would not exclude identification of other actinomycete genera as members of this family. As discussed (Stackebrandt et al., 1997), the presence of a pattern of 16S rDNA signature nucleotides common to all members of *Micromonospora* and related genera and different from those defined for other actinomycete families circumscribes the family Micromonosporaceae at the genomic level. Another set of signature nucleotides common to all monophyletic families led to the description of the order Actinomycetales, yet another set to the definition of the six actinobacterial orders of the class Actinobacteria.

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