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On the cover

Original waterolor of a bacteriology laboratory bench by Noel Krieg, 1955.

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Comprehending microbial diversity: the fourth goal of microbial taxonomy

James T. Staley

The primary goals of microbial taxonomy are Nomenclature, Classification and Identification. This paper proposes that a fourth goal, Comprehending Microbial Diversity, be included along with these three traditional objectives of bacterial and archaeal taxonomy. There are two major reasons for this. First, microbiologists have only recently begun to recognize that a vast, unknown diversity of microbial life exists on Earth. Therefore, there are many thousands or perhaps millions of species of *Bacteria* and *Archaea* that have not yet been discovered, cultivated and named. The second reason is that microbiologists are still debating the meaning of what comprises a bacterial species. Evidence from molecular analyses indicates that the current bacterial species is much broader than that of plants and animals therefore suggesting that many more microbial species, at levels below that of the current species, are not yet recognized. For this reason, microbiologists are encouraged to work with botanists and zoologists to develop a universal species concept that would unify biology and make the species taxon more uniform. This paper discusses these areas, the study of which will lead to a fuller and better comprehension of the true diversity of microbial life. Bergey's International Society for Microbial Systematics is an organization that will enhance the ability of the global community of microbiologists to work together to more fully comprehend the full extent of Earth's microbial diversity.

Introduction

The taxonomy of *Bacteria* and *Archaea* is viewed by some as an arcane branch of the academic sciences whose main goals are to provide names for new bacteria and archaea as well as a means of identifying and classifying them. The perception that microbial taxonomy is an esoteric, if not tedious, discipline is misleading for several reasons. From a mundane perspective, the names are used as a means to aid communication among researchers and the public alike. For example, *E. coli* is a species that is well known not only in the scientific community, but by the public as well. Microbial taxonomy also provides a means of identifying bacteria that are isolated from clinical and environmental samples. It is particularly important for clinicians who need to know which organism(s) is responsible for a particular infection some of which may not yet be recognized as pathogenic species. Third and foremost, microbiology as a science is built on a foundation of bacterial and archaeal species, whose identities (i.e., names and properties) are an essential aspect of understanding their biology. In that sense, microbial taxonomy is analogous to the chemical table of the elements: its ultimate aim is to provide, not only a logical nomenclature for each microbial constituent or species (i.e., the names of the "elements") but also a classification that shows how the bacterial and archaeal species are related to one another (Mendele'ev's "periodic table" of the elements). Therefore, microbial taxonomy enhances our scientific understanding of all the various branches of bacterial and archaeal life. However, unlike the periodic table of the chemical elements that can be displayed on a single page, comprehending the numbers of species of bacteria and archaea, their phylogenetic classification and the role(s) of each species in its natural environment is a challenge that is orders of magnitude larger and more complex. Thus, our current knowledge falls far short of the ultimate goal of completely comprehending the enormous diversity of microbial life. Whether or not this is an attainable goal is unclear; what is clear is that the challenges encompassing the exploration of Earth's microbial world offer unrivaled research opportunities for microbial taxonomists as well as those who are simply interested in life's boundaries.

One of the leading figures in microbial taxonomy in the 20th century was Samuel T. Cowan. In his classic paper on microbial taxonomy, he designated the three areas or goals of taxonomy as: Nomenclature, Classification and Identification (Cowan, 1970).

Although microbiologists are aware of these three classical

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goals of microbial taxonomy, we are just beginning to comprehend microbial diversity, the subject of this paper. Because understanding microbial diversity is so important in taxonomy, this paper posits that microbial taxonomy should include the additional fourth area or goal: Comprehending Microbial Diversity. Comprehending Microbial Diversity can be separated into two areas that are relevant to bacterial and archaeal taxonomy, exploring microbial diversity and understanding speciation.

Exploring microbial diversity

A major breakthrough in biology was the discovery of the universal Tree of Life made possible by Carl Woese's research on the sequence of ribosomal RNA of the small-subunit of the ribosome (Woese, 1987). The basis for using sequence analyses of genes and proteins for discovering the evolutionary relatedness among organisms is derived from the work of Zuckerkandl and Paulding (1965). Sequences from the 16S rRNA of representative *Bacteria* and *Archaea* and the 18S rRNA from a variety of eukaryotic organisms revealed for the first time a scientifically based Tree of Life (Woese et al., 1990). The tree showed that there were three main lines of descent: the *Bacteria*, the *Archaea* and the Eukarya. In addition, one of the most striking revelations of the Tree of Life is that most of the genetic diversity of life it revealed is found among the microbial groups, the *Bacteria*, *Archaea* and Eukaryotic microorganisms. This finding is consistent with other evidence from fossils and biomarkers from ancient sedimentary deposits, that microorganisms were the first organisms on Earth so they were, and still are, the foundation of Earth's biosphere (cf. Staley and Reysenbach, 2003).

Since the discovery of the Tree of Life, microbiologists have used 16S rRNA gene sequencing to examine in more detail the diversity of the *Bacteria* and *Archaea* that have been isolated in pure culture. Furthermore, by extracting DNA from environmental sources, and using the polymerase chain reaction with conserved primers for the 16S rRNA gene, microbiologists have found many additional "potential" species that remain to be isolated and described. Even more exciting is the discovery of unexpectedly divergent major groups, i.e. representatives of bacterial and archaeal phyla, that were not previously suspected (e.g., Hugenholz, 1998) and serve as targets for exploration by microbial taxonomists and ecologists.

The RDP (Ribosomal Database Project), which began with fewer than 1000 16S rRNA gene sequences in 1992, contained in excess of 670,000 annotated sequences in 2008 (Cole et al., 2009), illustrating the enormous growth of our understanding of microbial diversity. Since many of these sequences are from environmental sources and not from named pure cultures, they provide evidence of our increasing understanding of diversity, and a hint of the magnitude of the diversity that resides in the biosphere.

In addition, microbiologists have recently begun to seriously consider the distribution and mass of microbial life on Earth. Whitman et al. (1998) showed that the extent of Earth's biosphere is still largely unknown due in particular to organisms that reside within the crust and deep sediments. Based on estimates available at the time, the authors concluded that at least half of the biomass on Earth was microbial.

Clearly the recognition that more than half of the biomass is microbial is an important step toward understanding it. When this information is coupled with our knowledge about the diversity of microbial life as shown by the 16S rRNA surveys, it confirms our lack of a comprehensive understanding of the biosphere, in particular that part of it in which only microbial live exists.

The traditional approach to understanding novel organisms begins with the isolation of pure cultures. Considerable progress has been made using the classical procedures for enrichment and isolation of pure cultures, followed by determination of their 16S rRNA gene sequence, and a description of their phenotypic features. These are all part and parcel of the job of the microbial taxonomist, whose goal is to name them, understand their properties and deposit them in culture collections to make them available to other microbiologists.

Beyond the traditional approaches, novel and imaginative approaches will be needed to isolate bacteria and archaea from more unusual habitats where much of the unknown diversity resides. A new species from one of the unknown and uncharacterized phyla is occasionally isolated and the information obtained from its description may be useful in designing enrichment and cultivation media to isolate additional members. Of course not all of these organisms will be readily cultivable so, as straightforward as the approach may be, it will require considerable labor, and in many instances it will not be possible to predict whether a particular approach will work.

Another major technological breakthrough has been the development of genome sequencing. Genome sequences are providing detailed information on the complete genetic composition of *Bacteria* and *Archaea*. Even without knowing the phenotypic properties, one can examine the entire gene sequence of a strain, which in a sense is the chemical

formula for the organism, and learn much about an organism's potentially expressible properties, or phenotype.

The metagenomic approach, in which all extractable DNA from a natural sample is sequenced and subjected to genomic analyses, has also been successfully applied to elucidate the genetic diversity of some habitats (Tyson et al., 2005; Venter et al., 2004). This approach, especially when it is coupled with the genome sequences of representative strains from the community can be very informative.

Understanding microbial speciation

Surprisingly, considering that microbiologists have a definition for a species and more than 7000 described species have been named, microbiologists are still undecided about what a bacterial species is. Currently bacteriologists use a polyphasic definition that relies on two different types of analyses: phenotypic traits and DNA–DNA hybridization (DDH). This has served microbiologists very well since for more than 20 years (Wayne et al., 1987) in that it has stabilized bacterial taxonomy and made species more uniform among different taxa.

Recently criticisms have been made of the current polyphasic species definition. These fall into two general categories:

Bacterial species definition is neither conceptual nor natural

A major criticism of the polyphasic species definition is that it is not conceptual but instead is based on a combination of two types of information: data collected on phenotypic properties and molecular analyses of DNA–DNA hybridization (DDH) between two strains that are being compared. Although the polyphasic approach is a workable and practicable, it is not based on the natural processes whereby bacteria evolve to produce new species.

Recently, several proposals have been made for alternative ideas or concepts for bacterial species (Achtman and Wagner, 2008; Rosselló-Mora and Amann, 2001; Staley, 2006; Ward, 2006). These and other proposals for different concepts should be viewed as a healthful development as they indicate microbiologists are beginning to think of alternative ways of viewing what comprises a microbial species. Out of these possibilities a new species concept that is acceptable to bacterial taxonomists may be forthcoming.

Many of these new proposals use DNA and protein sequence analyses as a means of assessing speciation. A popular approach that has been pursued by many is the use of multiple locus sequence typing or analysis (MLST or MLSA) in which analyses of the sequences of several core genes or proteins (typically from 5 to 8) are concatenated and then subjected to phylogenetic analysis. This approach has been successfully applied for identifying members of some well-known genera including *Neisseria* and *Streptococcus* (Hanage et al., 2006).

Some have expressed concern about the impact that horizontal gene transfer (HGT) has on speciation (Doolittle and Bapteste, 2007). Clearly if HGT were too extensive, it would obscure the evolutionary record especially at the species level where HGT is more frequent than at higher taxonomic levels. If so, that would mean that there would be a serious if not fatal flaw for any species concept. However, there is no convincing evidence for such extensive HGT for most species. A recent excellent review of this and other issues presented here can be found in Cole et al. (2010).

Bacterial species definition is too broad

Another criticism of the polyphasic species definition is that bacterial species are more broadly defined than animal and plant species based upon molecular criteria (Cohan, 2002; Konstantinidis and Tiedje, 2005; Staley, 1997). For example, the cutoff for a bacterial species based on DDH is >70%. DDH values above this are considered to be members of the same species, whereas those below are considered as different species. If this definition were applied to primates, then all primates from lemurs to humans would be considered as the same species, which is absurd (Staley, 1997). Moreover, because of this broad definition, which is based on a standardized DDH cutoff, many potential bacterial and archaeal species have not been recognized.

One of the best examples that illustrates just how broad the bacterial species definition is, is that it has been very difficult to detect bacterial endemism in natural environments. At this time only one example can be cited of an archaeon or a bacterium that is actually endemic. The example that has been shown to be endemic is the archaeon *Sulfolobus islandicus*.

The initial study used about 70 strains of *S. islandicus* that were isolated from hot springs from Iceland, North America (Yellowstone National Park and Lassen National Park), and Russia's Kamchatka Peninsula (Whitaker et al., 2003). When the 16S rRNA gene was analyzed, there was no indication of any geographic clustering. However when MLSA was applied using eight concatenated genes, the strains from Iceland, North America and Russia were distinctly grouped into geographic clusters. Furthermore, although the hot spring strains from the two North American sources, Yellowstone and Lassen, which were clustered near one an-

other, were in distinguishably separate clusters. Importantly follow-up work using genome sequences of representatives from three different geographic areas even more clearly confirmed the clustering is real indicating that some prokaryotic microorganisms are endemic (Reno, 2009).

Is a universal species concept attainable?

This period during which microbial species concepts are being contemplated, also provides an opportunity for microbial taxonomists to work with taxonomists in botany and zoology to determine whether it is possible to agree on a species concept that is universally applicable to all organisms. Although this might seem like an impossible goal, if it were successful it would help unify the entire biological community (Staley, 2009). Furthermore, it would make the species taxon more comparable and uniform among all organisms.

Coda

In summary, microbial taxonomy is a field that is blessed with an exciting future: a biospheric cornucopia of microorganisms that is ripe for harvesting and from which microbiologists can more fully discern the taxonomic, genetic, physiological, ecological and biotechnological richness of microbial life. Bergey's International Society for Microbial Systematics is an organization whose primary goal is to work with the global community of microbiologists to more fully comprehend this cornucopia of Earth's microbial diversity.

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The importance of phenotype for bacterial systematics

Peter Kämpfer

Over the last 30 years, the characterization and identification of prokaryotes has undergone a dramatic change. Nowadays, the first step in characterization is often the determination of the 16S rRNA gene sequence. Based on a computer-generated comparison of the 16S rRNA gene sequence with sequences from reference strains, it is possible to allocate an unknown to a taxonomic group, often to a genus, rarely to a species. Isolates, which may represent novel taxa (genera or species) are then further characterized in order to find additional markers which are different from those reported for already established taxa. In several cases, only a very restricted set of phenotypic differences is reported and hence the classification of novel taxa is based largely on the 16S rRNA gene sequence differences. Sometimes additional (housekeeping) gene sequence differences are also reported, which may be regarded as sufficient for the delineation of novel species or even genera. There is a current trend to delineate bacterial taxa more and more on the basis of the genotype. But only the phenotype shows what genetic information is expressed and hence the phenotype represents an additional important level of information. In the case of a strain or set of strains which may represent novel taxa, it is therefore still essential that they are characterized both genotypically and phenotypically as comprehensively as possible. Only the interplay between genetic and phenotypic data sets may provide a basis for an in-depth taxonomy of the prokaryotes.

Introduction

In the last 20 years the numbers of proposals of novel prokaryote genera and species have increased enormously. This is due to the fact that more detailed studies on prokaryote communities in all kinds of environments are now possible, because of a growing set of molecular methods which is leading to a deeper insight into the tremendous diversity of prokaryotes. Moreover, it has become increasingly easy to perform sequence analyses of genes and even genomes, leading to an enormous amount of sequence data.

It has been widely accepted, that the 16S rRNA gene sequence serves as the "backbone" of bacterial systematics and for this reason, the first step in characterization of an unknown isolate is often the determination of the 16S rRNA gene sequence. On the basis of computer-generated comparisons of this sequence with sequences from reference strains available in databases, it is relatively easy to allocate an unknown to a taxonomic group. The description of a new genus is often based on low 16S rRNA gene sequence similarities (<95–97%) in comparison to sequences of established genera. Similarly, the description of novel species starts also with the detection of low 16S rRNA gene sequence similarities (< 98%) to gene sequences of established species.

Subsequent investigations include studies on basic phenotypic traits, such as cell and colony morphology, Gram-staining behaviour, physiology and biochemistry and studies on cell constituents, such as cell-wall, and / or membrane components. But these data are not always given; sometimes only literature data are reported, and they are not always sufficiently considered in the proposals for novel taxa. In practice, we have the "16S rRNA gene-based approach" (often called "Phylogenetic approach") rather that a true "polyphasic approach". Polyphasic taxonomy refers to classifications based on a consensus of all available methods: including phenotypic and genomic data (Colwell, 1970). Two selected examples shall illustrate why a comprehensive genotypic and phenotypic characterization is still necessary in order to come to a conclusive and stable classification.

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Example 1: The genera *Massilia* and *Naxibacter*

The genus *Massilia* was proposed in 1998 by La Scola et al. (1998), who described this new genus on the basis of one isolate from clinical material. At the time the 16S rRNA sequence similarity to the most closely related genera *Duganella* and *Telluria* was in a range of 92.9–94.6%. The descriptions were based on the 16S rRNA gene sequence data, some morphological, physiological test results and fatty acid composition. Unfortunately, no comparative chemotaxonomic data were presented, but the formal requirements for validation of the name were clearly fulfilled and the name was validated in 2000 (Validation List no. 73, 2000). In subsequent years, a total of 11 species isolated from various materials of this genus have been discovered and described (Gallego et al., 2006; Zhang et al., 2006; Zul et al., 2008; Weon et al., 2008; 2009; 2010). In 2005, Xu et al. proposed the related genus *Naxibacter* with the type species *Naxibacter alkalitolerans*. On the basis of 16S rRNA sequence similarity the genus was grouped in the vicinity of *Massilia*, *Telluria*, *Duganella* and *Janthinobacterium. Naxibacter* showed the highest similarity to *Janthinobacterium* (95.2–95.5%), followed by *Duganella* (95.2%) and *Massilia* (94.9%).

One very interesting characteristic of *Naxibacter alkalitolerans* was the report of the lipid phosphatidylinositol mannoside by Xu et al. (2005). This lipid is normally not detected in Gram-negative bacteria. In a later study, the presence of this compound could not be confirmed for other strains closely related to *N. alkalitolerans* (Kämpfer et al., 2008) and also not for the type strain of *N. alkalitolerans*.As a consequence the genus description was emended (Kämpfer et al., 2008). Later, Weon et al. (2010) could also not detect phosphatidylinositol mannosides in the polar lipid profile of *Naxibacter suwonensis*.

Thus, members of the genus *Naxibacter* and *Massilia* show very similar phenotypic traits. Both were characterized as aerobic, Gram-negative, motile, non-spore-forming rods. All had a fatty acid profile consisting of $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7*c*, $C_{18:1}$ω7*c*, $C_{16:0}$ and $C_{10:0}$ 3-OH as the characteristic fatty acids. They had Q-8 as their major ubiquinone and the major polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. They are clearly grouped together on the basis of 16S rRNA gene sequence analyses (Kämpfer et al., 2008; Weon et al., 2009; 2010, Figure 1).

In 1996, strain CCUG 58010^T was isolated in Göteborg, Sweden from the blood of a 48 year old man. This strain was presumptively identified as *Massilia–Naxibacter*-like, but on the basis of the16S rRNA gene sequence and also phenotypic data it was impossible to place it clearly in either *Massilia* or *Naxibacter*. As a consequence Kämpfer et al. (2010) proposed to include all *Naxibacter* species in the genus *Massilia* in order to have a consistent classification of the group. The taxonomic position of some *Massilia* species (*M. plicata*, *M. dura*, *M. albidoflava*, and *M. lutea*) is still unclear. They are grouped most closely to species of the genus *Telluria* and a comprehensive comparative phenotypic study should eventually allow a more in-depth view into the taxonomy of this group.

This example clearly illustrates the necessity for basing a genus description not solely on 16S rRNA gene sequence data but also on phenotypic properties. Murray et al. (1991) pointed out that "an important objective in proteobacterial systematics (but not only for proteobacterial systematics), therefore should be the thorough examination of representative strains from different branches to highlight appropriate chemical markers for the definition of higher taxa above the species level. ….It is completely impracticable to define genera solely on the basis of "phylogenetic" data. Genera need to be characterized by using phenotypic properties, even if the choice of phenotypic markers might change given the development of better tests" This is consistent with the view of Stackebrandt et al. (2002) who indicated that more emphasis should be placed on discriminating markers. As they noted, species should be based on the use of well-documented criteria, laboratory protocols and reagents that are reproducible (Stackebrandt et al., 2002). In practice descriptive and diagnostic characters should be described in sufficient detail to permit comparisons among taxa and allow reproduction of observations (Stackebrandt et al., 2002). It should be mentioned here, that this is only one selected example showing the importance of a polyphasic approach in the sense of the definition of Colwell (1970). There are numerous other examples that bacterial genera are unambiguously defined phenotypically (e.g. most of the genera of the families *Enterobacteriaceae*, *Vibrionaceae*, *Rhizobiaceae*, just to name a few well known examples). It is also important to notice that for the description of a novel genus, phenotypic data have to be provided which clearly characterize the genus and which should preferably be more "conserved" than those traits useful for species discrimination. Genus-specific traits should be those which could be expected to be shared by future members of this genus. On the basis of 30 years experience with chemotaxonomic data, the analyses of polar lipids profiles, quinone types and polyamine- and fatty acid patterns, these data may be suitable for this purpose and it has been shown in numerous publications that there is a strong correlation between the 16S rRNA gene based classification and chemotaxonomic features.

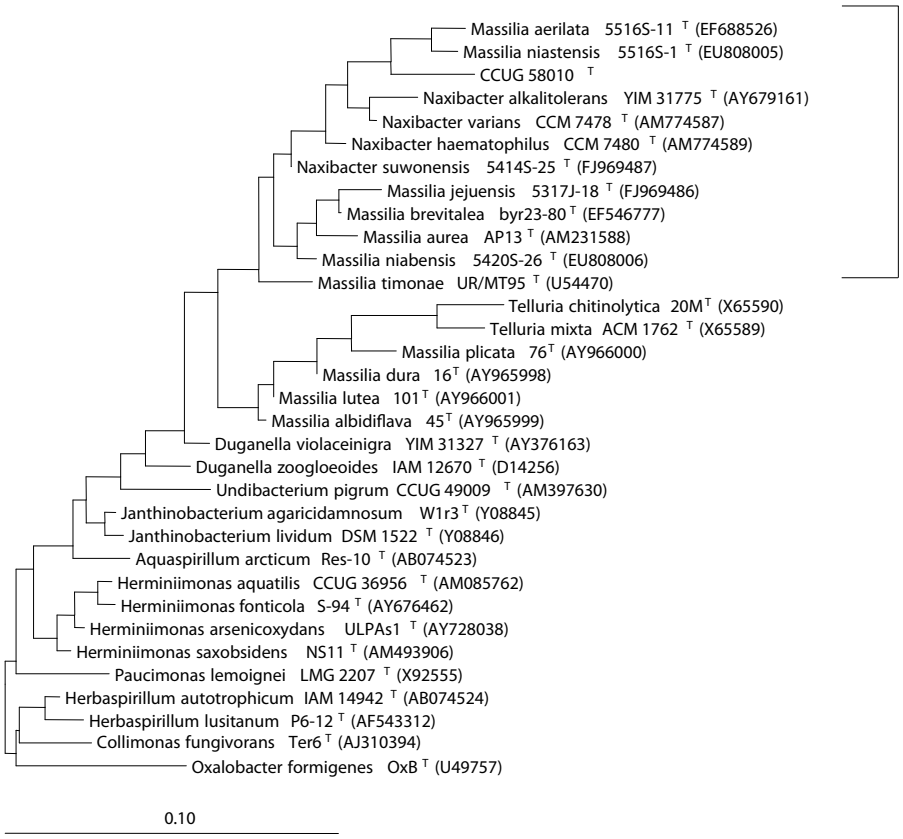

Figure 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the European Molecular Biology Laboratory data library (accession numbers are given in parentheses). The phylogenetic tree was constructed using the ARB software package, after multiple alignment of data with the ARB alignment tool and the SILVA SSURef 100 database (release August 2009). Tree building was performed using the maximum likelihood method with fast DNAml without filters. Bar, 0.10 nucleotide substitutions per nucleotide position.

Example 2: The *Pseudomonas fluorescens* group

A most comprehensive characterization is also necessary for the description of novel species. Stackebrandt et al. (2002) provided an open "definition" of the species as "a category that circumscribes a (preferably) genomically coherent group of individual isolates /strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions.

Despite the well known and documented methodological problems of the DNA–DNA hybridization techniques (for details see Rosselló-Mora, 2008), DNA–DNA similarity remains the acknowledged standard for species delineation (Stackebrandt et al., 2002), although it was already recognized at that time that multilocus sequence typing (MLST) using sequence analysis of housekeeping genes had brought a new dimension into the elucidation of genomic relatedness at the inter- and intraspecific level.

Hence, Stackebrandt et al. (2002) encouraged studying the role of DNA sequence data in classification from protein-coding genes as possible molecular criteria for species delineation. Furthermore, sequence analysis of complete genomes was envisaged as enabling the analysis of genes across a wide range of bacteria. The study of "housekeeping" genes has become increasingly important, for both classification and identification, but even here there are some difficult problems that must be addressed. For example, it is often still not clear which housekeeping genes are suitable. Only a limited number of genes occur in all genomes. Furthermore the information content of these genes for "phylogenetic" analyses is often not clear. Recognition of paralogous genes is still a problem, and sequence analysis of paralogous housekeeping genes often leads to conflicting tree topologies.

The following example illustrates, why a detailed look at the interpretation of sequence analyses of housekeeping genes

is essential, and why a comprehensive genotypic *and* phenotypic characterization is necessary for species delineation in order to come to a conclusive and stable classification.

The number of *Pseudomonas* species closely related to *P. fluorescens* has increased enormously. Anzai et al. (2000) reported on the high 16S rRNA gene sequence similarity of several species of the *Pseudomonas fluorescens* group, *P. marginalis*, *P. rodesiae*, *P. lurida*, *P. libanensis*, *P. gessardii*, *P. cedrina*, *P. azotoformans*, *P. synxantha* and *P. mucidolens*. Additional species of this group, *P. poae*, *P. trivialis; P. extremaustralis* and *P. brenneri* were described later and the type strains of these species show 16S rRNA sequence similaries ranging from 98.3% to 100% (Figure 2). The genus *Pseudomonas* is chemotaxonomically homogeneous and no significant phenotypic differences in the polar lipid or polyamine patterns, the quinone type and the fatty acid profiles have been published. In addition, the physiological/biochemical test results of the type strains of these species are very similar, and because the majority of these species were proposed on the basis of only one strain, an intraspecific physiological/biochemical variability has not been reported. Stackebrandt et al. (2002) emphasized that more emphasis should be placed on discriminating markers, but recommended also, that sequence analysis of housekeeping genes should be investigated in order to find additional markers.

Several housekeeping genes for type strains of these species have been investigated, among them *gyrB*, *rpoD*, *fusA*, *recA*, among others. A MLST database has been recently set up by Bennasar et al. (2010); however, in this case the interpretation of the information "behind" the sequence data is important. A comparison of the *gyrB* sequences of the type strains of the *Pseudomonas* species shown in Figure 3 showed similarities of 87.8% to 99.9% (if all nucleotides are considered). The amino acid sequence data (Figure 4) revealed much higher similarities of 98.3–100%. The same situation was found for the *fusA* sequence similarites (ranging from (88.3–98.7%) in comparison to the amino acid sequence similarities (94.8–100%, Figure 5) as well as for the *recA* and *rpoB* gene sequence data in comparison to the amino acid sequence data. These examples show clearly that sequence data have to be interpreted very carefully as similarity at the phenotypic level (here the amino acid sequence is regarded as the phenotype) is very much higher, than the underlying genotypic data. Because of the high 16S rRNA gene sequence similarities, the high degree of phenotypic similarities (shown by classical phenotyping, i.e. physiological tests) and the amino acid sequence data of housekeeping genes, some of these species might be combined in a single taxon. The DNA–DNA hybridization distances among the type strains of the species shown in Figure 2, repeated according to the method of Ziemke et al. (1998) also show relatively high values in the range of 60–80% (unpublished data). In addition, more than 90 out of 100 physiological tests (Kämpfer et al., 1991) performed with all type strains showed identical results (unpublished data). Therefore, the inclusion of several species as shown in Figure 2 seems to be a logical approach.

In regard to sequence data, Ludwig and Klenk (2001) previously pointed out, that the information content for the 16S rRNA gene is restricted by functional constraints, only 972 (63.2%) variable (informative) positions are available in the *Bacteria*, and 971 (63%) in the *Archaea*. Only 407 (26.4%, *Bacteria*) and 301 (19.5%; *Archaea*) positions are found for which all 4 nucleotides are allowed. The information content of protein coding genes is even more restricted. A detailed analysis of the *rpoB* gene for taxonomic purposes has been provided by Adékambi et al. (2008). While it is obvious that these sequence data are very helpful and useful in taxonomy, especially the identification of different bacterial genera and species, their use in classification should always be accompanied by other data (as shown above).

It should be briefly mentioned here, that concatenation of data may lead to additional problems and incorporation of variation in gene histories into multilocus phylogenetic analyses is necessary. As pointed out by Salter Kubatko and Degnan (2007), concatenation of sequences from multiple genes prior to phylogenetic analysis often results in inference of a single, well-supported phylogeny. Theoretical work, however, has shown that the coalescent can produce substantial variation in single-gene histories. Using simulation, Salter Kubatko and Degnan (2007) examined the performance of the concatenation approach under conditions in which the coalescent produces a high level of discord among individual gene trees and showed that it leads to statistically inconsistent estimation in this setting. In addition they found, that the use of the bootstrap to measure support for the inferred phylogeny may lead to a moderate to strong support for an incorrect tree under these conditions.

In general, the mode of comparison of sequence data sometimes leads to confusing interpretations. The term "phylogenetic" is associated with a number of meanings. In the majority of papers describing novel genera or species, "phylogenetics" has become associated with the study of gene or protein sequences. Sneath (1989) has indicated the problems with an uncritical use of the term and he noted, that it is not easy to determine when "phylogenetic" means evolutionary, cladistic, or simply genomic. As recommended by

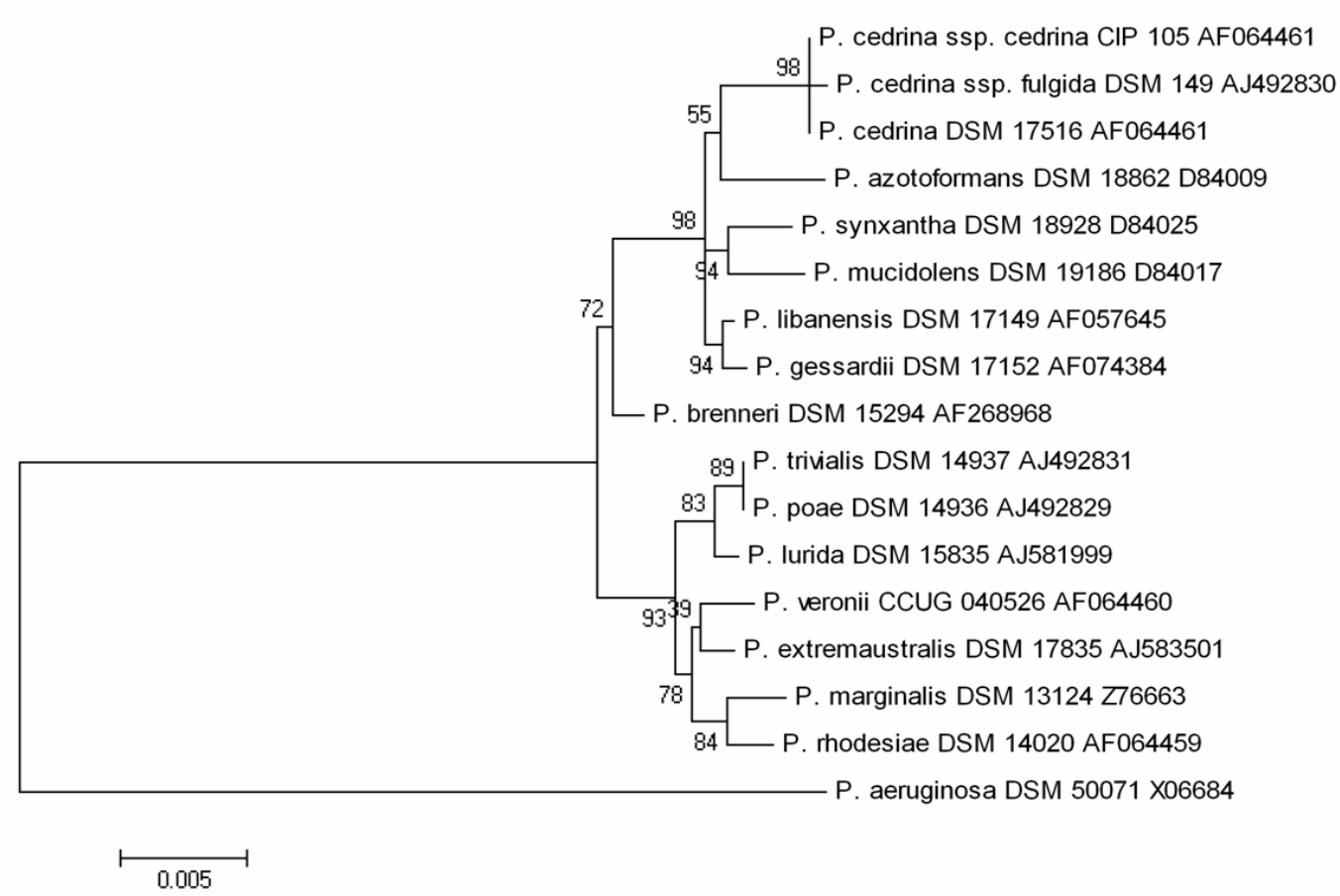

Figure 2. 16S-rRNA gene sequence comparison. The tree was calculated using the Neighbor-Joining method]. The optimal tree with the sum of branch length = 0.09434948 is shown. Similarities among investigated species: 98.3–100% The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (1000 replicates is shown next to the branches]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1453 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

Tindall et al. (2008), it is probably best to avoid those terms which cause confusion altogether and simply divide the methods of classification into three: Overall similarity, "character analysis", and a combination of both.

For comparison of sequences, numerical taxonomic methods are used; however, the term "numerical taxonomy" in microbiology is often restricted to mean "the use of computers for the comparison of phenotypic data". But the basic elements of numerical taxonomy are evident in the vast majority of alignment programs and in the principle underlying simple BLAST searches. BLAST searches determine the most similar sequence(s) in the database without being able to confirm to what extent the sequences being compared represent the same proteins or genes (i.e., homologues). As already pointed out by Sneath (1989), sequence data are often referred to as phylogenetic rather than phenetic, but this is not so. It is doubtful whether data can be called phylogenetic, because the term properly applies to relationships, and hence depends on methods of analysis.

In summary, the description of novel genera and species require the careful selection and use of a wide variety of methodologies.

It is an interesting development, that, although the 16S rRNA gene sequence has been widely accepted as the "backbone" of bacterial systematics as part of the often called "tree of life", in the light of genome data the concept of a single universal tree of life appears increasingly obsolete, especially in the light of the impact of lateral gene transfer events (see e.g. Bapteste et al., 2009; Boucher and Bapteste, 2009; Da-

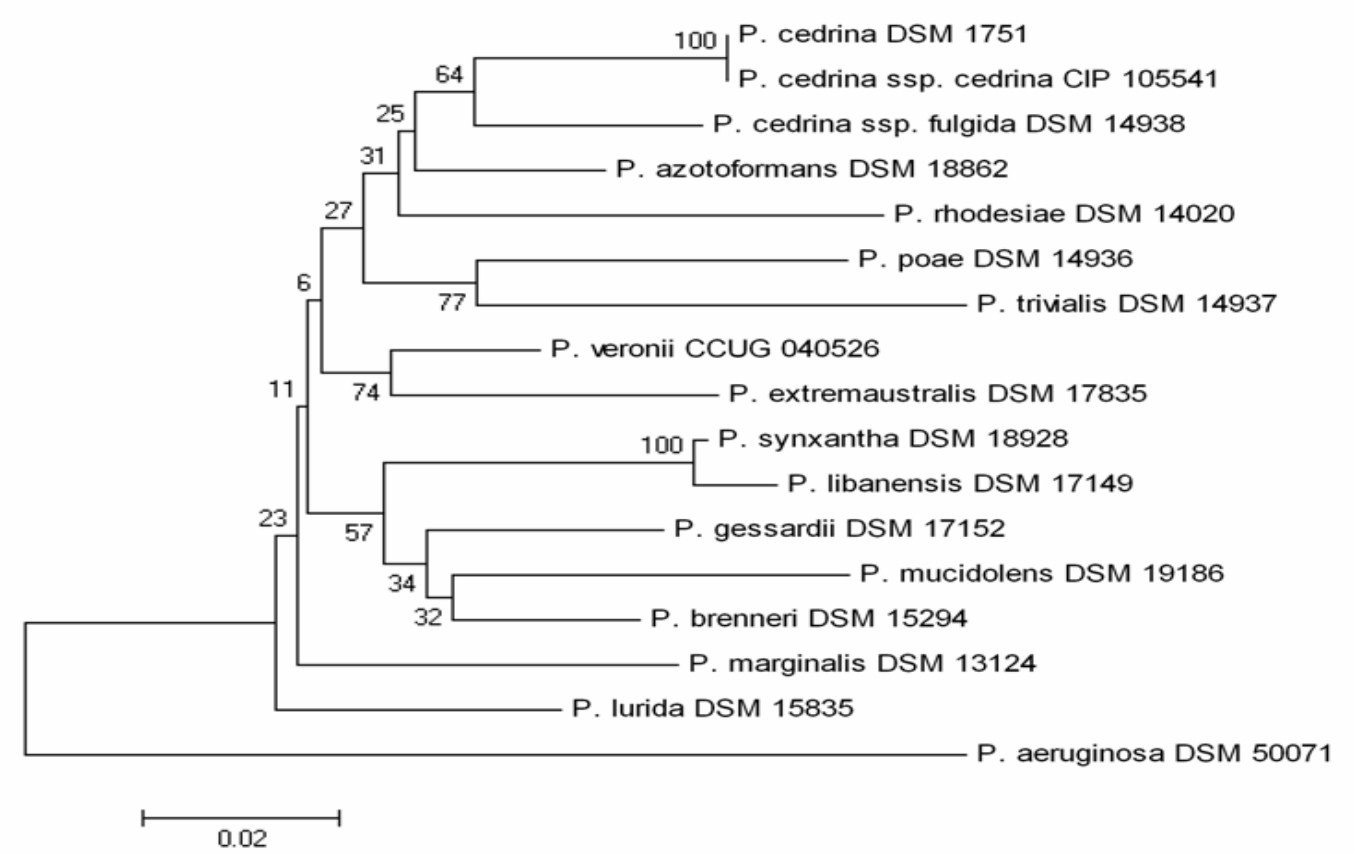

Figure 3. Relationship of *gyrB* gene sequences. The analysis was performed using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.62147606 is shown. Similarities: 87.8–99%. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 513 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

gan et al., 2008; Fourier and Gogarten, 2010; Fourier et al, 2009; Kreimer et al., 2008; Wolf et al., 2002 and others for a more detailed discussion), however, it should be mentioned here, that methodologically it is hard to infer HGT unless one has an *a priori* hypothesis of relationship that indicates the presumptive transferred genes are not homologues.

The availability of more and more genome data has stimulated a debate whether evolutionary relationships between prokaryotes may be best regarded as a tree or a network. But independent from these debates and despite the advantages of molecular methods (including the generation and analyses of whole genome sequence data) it is often impossible to deduce phenotypic properties from the presence or absence of genes and gene clusters, because genes do not exist for their own sake. This is especially true for seemingly simple, but nevertheless "complex" phenotypic properties, like temperature, NaCl or pH tolerance (just to name a few), which may be affected by very different and complex regulatory biochemical networks, which are based on the underlying genetic potential and expression network.

As pointed out by Tindall et al. (2010), experience gained over the past six decades has continued to demonstrate the value of comparing different datasets and also of basing the description and delineation of taxa on as wide a dataset as possible. Only a combination of data acquired from DNA-based methods (DNA–DNA hybridization, gene sequences, genomic fingerprints) and phenotyping (chemotaxonomic, physiological and morphological traits) provides a sound basis for the taxonomy of the prokaryotes (Tindall et al., 2010).

Molecular data can provide an enormous amount of information. However, at this point, we are far from able to interpret these data (especially the information behind them well

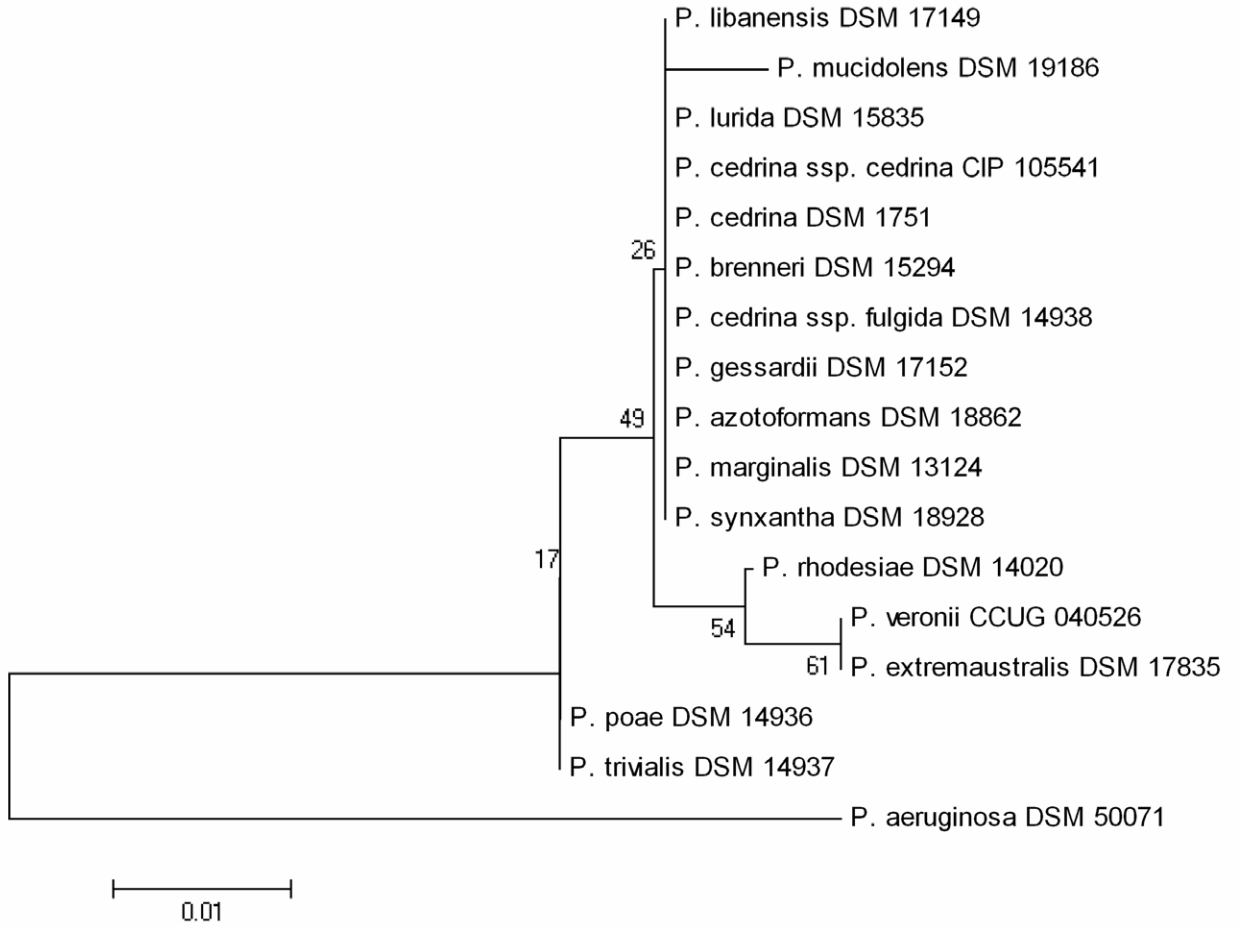

Figure 4. Relationships of *gyrB* amino acid sequences. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.10028940 is shown. Similarities: 98.3–100%. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 171 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

enough to draw decisive conclusions. There are numerous open questions, e.g. "Which genes belong to the conserved genome core and are considered likely useful to define a taxon and which belong to accessory dispensable genetic elements?´ The "overall" impact of processes such as lateral gene transfer, gene duplication, recombination and rearrangements of genes in the genome is not clear and may vary considerably in different lineages (see Bapteste et al., 2009; Dagan et al., 2008 and other publications). In addition, the presence of genes and gene clusters (whether expressed or silent) can have a totally different biological meaning, and the roles of structural elements (some of them phenotypically recognizable by the so-called "chemotaxonomic" methods) and biochemical pathways (also recognized by studying the phenotype at different levels) should be consistent with the underlying genetic data. which is essentially the aim of a "polyphasic taxonomy" and the basis of the presentation of genera and species in *Bergey's Manual of Systematic Bacteriology,* 2nd edn (Boone and Castenholz, 2001; Brenner et al., 2005; De Vos et al., 2009; Krieg et al., 2010; Goodfellow et al., 2011).

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Description of new yeast species – is one strain enough?

Cletus P. Kurtzman

The issue of description of new yeast species on the basis of a single strain is discussed. Single gene sequences, such as those from D1/D2 LSU rRNA, or sequences from ITS1/ITS2 are commonly used as the basis for recognizing new yeast species. Evidence is presented that hybrids and species with polymorphic gene sequences may not be recognized from a single gene analysis, but with multigene sequence comparisons, single-strain species can be accurately determined. Further, description of single-strain species will add to an understanding of yeast phylogeny and species diversity, which would be unknown if new species descriptions were limited to those taxa for which multiple strains were available.

Introduction

Quantification of gene sequence divergence among taxa as a means to identify microbial species is now commonplace for bacteria, yeasts and other microbial groups (Kurtzman and Robnett, 1998; Stackebrandt et al., 2002; Taylor et al., 2000). With reliance now on a genetics-based system of identification rather than on phenotypic methods, which often can only be interpreted subjectively, we are able to re-examine the question of whether the description of new species should be based on a single strain if only one isolate is available. It should be noted, however, that although identification of species from phenotype often proves unreliable, inclusion of phenotypic data in a species description provides information on the physiology and genetic properties of the organism.

Arguments for requiring multiple strains for a species description include the need to understand genetic diversity within the species, the extent of phenotypic variation among strains and an understanding of the ecology of the new species. Arguments for describing a new species based on a single available strain include recognizing additional new germplasm that may be of importance to science and biotechnology and providing a better understanding of the phylogeny and biodiversity of the taxonomic group to which the species will be assigned.

Of the approximately 700 species presented in *The Yeasts, a Taxonomic Study*, 4th edn (Kurtzman and Fell, 1998), nearly one-third (30%) were described from a single strain. This same trend is shown for the nearly 1500 species treated in the 5th edition of this book (Kurtzman et al., 2011). Species included in both of these taxonomic treatments were circumscribed from molecular comparisons and appear to represent biologically distinct taxa. Therefore, their exclusion would remove approximately one-third of known yeast biodiversity. However, some would still argue that description of single-strain species is a poor practice (e.g., *Yeast Newsletter*, December 2002, p. 77, http: //publish.uwo.ca/~lachance/Zy00492.pdf). In this review, some of the reasons why single-strain species descriptions should be made and some of the detrimental aspects of doing so will be discussed. Perhaps the major concern is whether species can be reliably resolved. In the following section, some of the molecular parameters used to recognize yeast species will be described and their limitations will be discussed. Following this, some of the other objections to single-strain species descriptions will be examined.

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Recognition of species from gene sequence divergence

Single-gene species resolution

Quantification of genome similarity became possible with the development of DNA reassociation techniques that measure the extent of pairing of nucleotide sequences between strains when the DNAs of two isolates are made single-stranded, mixed and allowed to repair as a double strand. An interpretation of DNA reassociation data was provided by Martini and Phaff (1973) and Price et al. (1978), who suggested that on the basis of shared phenotype, strains that showed 80% or greater nuclear DNA relatedness, as measured by reassociation, are members of the same species. Correlation of DNA relatedness with the biological species

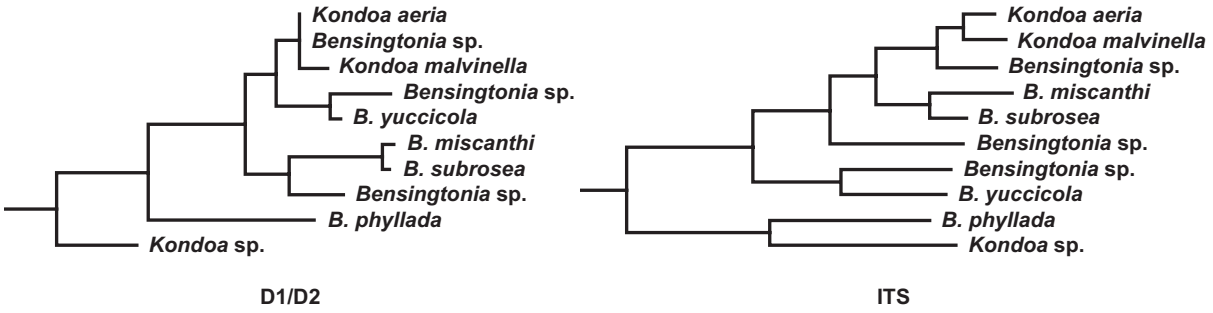

Figure 1. Contrasting resolution of *Bensingtonia* and *Kondoa* species when analyzed from gene sequences of D1/D2 LSU rRNA and from sequences of ITS. Lineages in this clade are more highly resolved from ITS sequences than from D1/D2. Adapted from Scorzetti et al. (2002).

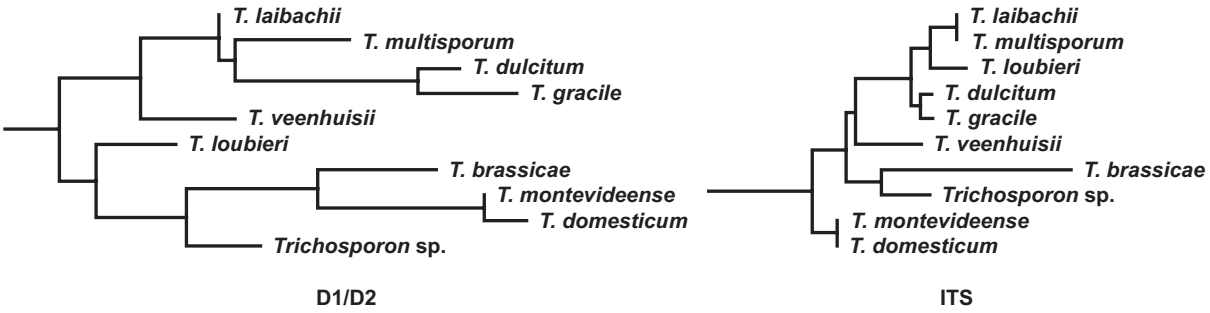

Figure 2. Resolution of *Trichosporon* species from analysis of D1/D2 LSU rRNA gene sequences and from ITS sequences. Greater species resolution is provided by D1/D2 than from ITS, which is in contrast to resolution of species in the *Kondoa* clade (Figure 1). Adapted from Scorzetti et al. (2002).

concept has been examined from genetic crosses utilizing both heterothallic and homothallic species (Kurtzman, 1984, 1987; Kurtzman et al., 1980a, b; Naumov et al., 2000; Smith et al., 2005; Vaughan-Martini and Kurtzman, 1985). In general, these studies, which included species assigned to the genera *Pichia*, *Lindnera*, *Saccharomyces* and *Zygoascus*, support the idea that strains showing 70–80% or greater DNA complementarity are conspecific. A limitation of DNA reassociation experiments has been that genetic resolution extends no further than to closely related species. In contrast, gene sequence comparisons offer the opportunity to resolve closely related species, as well as more distantly related taxa, and a database of sequences can be developed and continually expanded as new species become available. Such a database represents a barcode for species. Despite the limited resolution offered by DNA reassociation experiments, these data provided some of the initial reference points for interpretation of the gene sequence comparisons described below. However, with the present-day ease of obtaining gene sequences, DNA reassociation data are less often determined. A primary reason is that reassociation data appear less informative than analyses from gene sequences.

The variable domain 2 (D2) from nuclear large-subunit rRNA (LSU rRNA) appears to have been the first gene sequence examined for resolution of closely related yeast species. Using data from genetic crosses and the above discussed DNA reassociation experiments, Peterson and Kurtzman (1991) showed that even closely related yeasts could be resolved from differences in the D2 domain. Kurtzman and Robnett (1998) expanded this work to include domains 1 and 2 (D1/D2) of the LSU rRNA gene and determined the D1/D2 sequence for all described species of ascomycetous yeasts, resulting in a diagnostic database for rapid species identification. Fell et al. (2000) developed a complementary D1/D2 database for known basidiomycetous yeasts. By comparing divergence among ascomycetous strain pairs with previously determined nuclear DNA reassociation values, it appeared that conspecific strains differed by no more than three nucleotides among the 500–600 nucleotides of the D1/D2 domains, whereas differences of six or more nucleotides (1%) indicated that the strains were different species (Kurtzman and Robnett, 1998). The preceding guidelines developed by Kurtzman and Robnett (1998), which also seem to apply to basidiomycetous yeasts, were treated as a prediction because of the exceptions that will be discussed.

Sequences of the internal transcribed spacers 1 and 2 (ITS1 and 2), which are located between the nuclear SSU and LSU rRNA genes of the rDNA repeat, and separated by the

Table 1. Percentage nuclear DNA relatedness and LSU D1/D2 nucleotide divergence among closely related species of the *Meyerozyma* (*Pichia*) *guilliermondii* clade[a]

	M. guilliermondii		*M. caribbica*		*C. carpophila*	
Species	%DNA	D1/D2	%DNA	D1/D2	%DNA	D1/D2
M. guilliermondii	100	0	37	3	55	1
M. caribbica			100	0	68	2
Candida carpophila					100	0

[a]Data from Vaughan-Martini et al. (2005). DNA reassociation values are a mean from five strain pairs of each species. All strains of each species had the same D1/D2 sequence.

Table 2. Extent of nuclear DNA reassociation and gene sequence divergence between closely related species in several ascomycetous genera

		Genes (substitutions-indels)[c]			
Species pair[a]	DNA reassoc. (%)[b]	D1/D2	SSU	EF-1α	MtSm
Lindnera (*Pichia*) *amylophila-L.* (*P.*) *mississippiensis*	25	2-2	4-0	19-0	
L. amylophila-L. (*P.*) *fabianii*		9-2	19-2	39-0	
L. mississippiensis-L. fabianii		7-0	15-2	51-0	
Lindnera (*Pichia*) *americana-L.* (*P.*) *bimundalis*	21	2-0	0-0	22-0	
Lindnera (*Williopsis*) *saturnus-L.* (*W.*) *mrakii*	52	1-0	0-0	12-0	
L. mrakii-L. (*W.*) *subsufficiens*	44	4-0	0-0	12-0	
L. saturnus-L. subsufficiens	56	5-0	0-0	12-0	
Pichia cactophila-P. pseudocactophila	34	11-8	1-3	14-0	0-0
P. cactophila-Candida inconspicua[d]		1-1	0-0	0-0	0-0
Pichia kluyveri-P. eremophila	66	7-1	5-0	26-0	1-0
P. kluyveri-P. cephalocereana	72	3-0	2-0	11-0	1-0
P. eremophila-P. cephalocereana	69	7-1	4-0	25-0	1-0
Pichia (*Issatchenkia*) *scutulata-P.* (*I.*) *exigua*	25	20-7	7-1	36-0	10-4
Starmera (*Pichia*) *amethionina-S.* (*P.*) *pachycereana*	65	8-10	5-3	12-0	
S. amethionina-S. (*P.*) *caribaea*	40	21-2	8-3	11-0	
S. pachycereana-S. caribaea	37	17-5	9-5	6-0	

[a]Species pairs are type strains. Genus names in parentheses were those used in the publication describing these results.
[b]Data are from Holzschu et al. (1983), Kurtzman (1984, 1991), Kurtzman et al. (1980a, b), Phaff et al. (1976, 1987, 1992), Shen and Lachance (1993).
[c]D1/D2, Domains 1 and 2, LSU rRNA; SSU, small-subunit rRNA; EF-1α, translation elongation factor-1α; MtSm, mitochondrial SSU rRNA (Kurtzman et al., 2008).
[d]From these data, *Pichia cactophila* and *Candida inconspicua* are considered to be conspecific.

highly conserved 5.8S rRNA gene, are commonly used to resolve species, often in conjunction with sequences from the D1/D2 LSU rRNA gene. The resolution provided by ITS sometimes exceeds that of D1/D2, but the reverse is also true. For example, ITS clearly provides greater resolution of *Bensingtonia* and *Kondoa* species (Figure 1), but species of *Trichosporon* are less well resolved by ITS (Figure 2).

Of rRNA/rDNA regions used for species identification, the intergenic spacer (IGS) appears the most substituted and offers the greatest resolution of closely related species and subspecific lineages. The IGS is comprised of two regions, IGS1 and IGS2, which are often separated by the highly con-

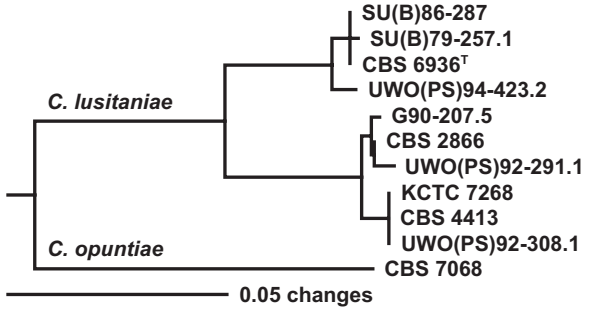

Figure 3. D1/D2 LSU rRNA gene tree demonstrating the sequence polymorphisms that were found among strains of *Clavispora lusitaniae*. T = type strain. Adapted from Lachance et al. (2003).

served 5S rRNA gene. IGS sequences have been used to resolve lineages within *Cryptococcus neoformans* and closely related taxa (Diaz and Fell, 2005; Diaz et al., 2000, 2005; Sugita et al., 2001) and for separation of closely related species of *Trichosporon* (Diaz and Fell, 2004; Sugita et al., 2002), *Mrakia* (Diaz and Fell, 2000) and *Xanthophyllomyces* (Fell and Blatt, 1999; Fell et al., 2007). A characteristic of IGS is the diversity of length polymorphisms. Sugita et al. (2002) reported that the IGS1 region ranged in length from 195–704 nucleotides among *Trichosporon* species. The IGS region often includes a series of multiple repeat units with numerous deletions and insertions (indels). These repeat units and indels provide characteristics for defining strains and species, which sometimes delineate the geographical distribution of strains (Fell et al., 2007; Libkind et al., 2007).

Genes other than those from the rRNA repeat have been examined for their capability to separate species. Daniel and Meyer (2003) and Daniel et al. (2001) compared species resolution from divergence in the gene sequences of D1/D2 LSU rRNA and actin-1. Divergence in actin-1 was greater than in D1/D2, thereby potentially providing greater resolution among closely related taxa. As was found for D1/D2, actin-1 sequences are sufficiently variable in nucleotide substitutions among apparently conspecific strains that it is sometimes difficult to resolve divergent strains from closely related species with just a single gene. In a comparison of *Saccharomyces* species, substitutions in ITS1–5.8S–ITS2 and the genes encoding D1/D2 LSU rRNA, translation elongation factor-1α, mitochondrial SSU rRNA and cytochrome oxidase II provided similar resolution (Kurtzman and Robnett, 2003).

Although the majority of yeast species appear to be reliably recognized from single gene sequence analyses, the guidelines presented by Kurtzman and Robnett (1998) for resolution of species from D1/D2 sequencing were treated as a prediction because exceptions had been found earlier. DNA reassociation studies revealed *Saccharomyces pastorianus* to have intermediate relatedness with *S. cerevisiae* (57%) and *S. bayanus* (72%) (Vaughan-Martini and Kurtzman, 1985), and *S. bayanus* and *S. pastorianus* were found to have identical D2 sequences (Peterson and Kurtzman, 1991). These results were interpreted to mean that *S. pastorianus* is a hybrid of *S. bayanus* and *S. cerevisiae* and that *S. pastorianus* received its D2 LSU rRNA gene sequence from *S. bayanus*. Later, *S. pastorianus* and *S. bayanus* were shown to share the entire rRNA repeat (Kurtzman and Robnett, 2003); consequently, these two sister species cannot be separated from sequence differences in D1/D2 rRNA, SSU rRNA or ITS. The problem of resolving hybrids was further illustrated by Groth et al. (1999) from the discovery that *Saccharomyces* sp. strain CID1 was actually a triparental hybrid with nuclear or mitochondrial DNA from *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii*.

The presence of indels further complicates the estimation of relatedness among strains when using single gene analyses. Liu and Kurtzman (1991) found 4–6 deletions in the D2 domain of LSU rRNA among strains of *Barnettozyma* (*Williopsis*) *californica*. The strains were believed to be conspecific because they showed 94–100% nuclear DNA relatedness as determined from reassociation (Kurtzman, 1991). The deletions were contiguous and may represent a single evolutionary event. Consequently, in the predictive guidelines offered by Kurtzman and Robnett (1998), contiguous deletions were treated as a single event and weighted as one nucleotide substitution. Similarly, Lachance et al. (2003) found certain strains of *Clavispora lusitaniae* to be highly polymorphic in the D1/D2 domains of the LSU rRNA gene (Figure 3). The polymorphic strains, which have similar actin-1 sequences, mated and formed ascospores, although ascospore viability was not determined. Further work is needed to understand the *C. lusitaniae* polymorphisms, and this would include comparing additional gene sequences as well as determining if there are multiple alleles of the LSU rRNA gene. Noteworthy is that Fell et al., 2007) reported sequence heterogeneity in the ITS and IGS regions among certain strains of *Xanthophyllomyces*.

Another factor influencing species resolution is the apparent difference in substitution rates among lineages for the diagnostic gene being used. For example, the closely related species *Meyerozyma* (*Pichia*) *guilliermondii*, *M. caribbica* and *Candida carpophila* differ from one another by 1–3 nucleotides in D1/D2 and will not be recognized as separate species using the guideline that 0–3 substitutions indicate conspecificity (Table 1). Examples of apparent lineage-specific differences in extent of substitutions for D1/D2 and certain other genes are given in Table 2. With the exception of the highly conserved SSU rRNA gene, each of the other genes listed resolves closely related species.

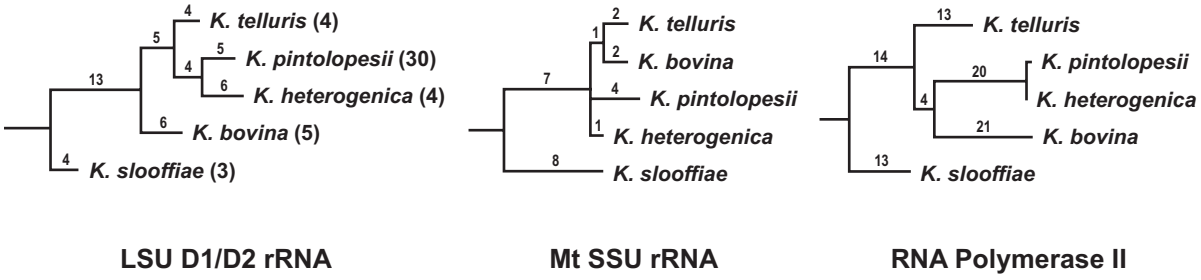

Figure 4. Maximum-parsimony analyses of gene sequences for D1/D2 LSU rRNA, mitochondrial SSU rRNA and RNA polymerase II from *Kazachstania* species. The gene trees show overall congruence, but for RNA polymerase II, *K. pintolopesii* and *K. heterogenica* are nearly unresolved, suggesting that the latter species is a hybrid. The mitochondrial SSU rRNA tree shows *K. telluris* and *K. bovina* to be more closely related than is indicated by the other two gene trees, again suggesting an interspecific hybridization event. Numbers above branches reflect nucleotide substitutions and numbers in parentheses are the number of strains sequenced for each species for all three genes. Adapted from Kurtzman et al. (2005).

Multigene species resolution

In the examples presented, determination of whether strains are conspecific or members of separate species can be confused by hybridization events (Figure 4), by unexplained sequence polymorphisms (Figure 3), and by differences in nucleotide substitution rates (Figures 1 and 2). Multigene analyses offer a means for detecting these changes, which would be signaled by lack of congruence for a particular gene tree. This approach was recommended by Goodman (1976) for vertebrates, for bacteria by Dykhuizen and Green (1991), and for filamentous fungi by Taylor et al. (2000). The paper by Taylor et al. (2000) provides an inclusive review of species concepts, and the term Genealogical Concordance Phylogenetic Species Recognition (GCPSR) was introduced to describe the concept of multigene analysis for species recognition. An example of GCPSR is found in a multigene sequence analysis of the *Kazachstania* (*Arxiozyma*) *telluris* species complex. D1/D2 LSU rRNA gene sequence analysis resolved the complex into five species as did analysis of mitochondrial SSU rRNA gene sequences. However, analysis of RNA polymerase II (Figure 4) detected only four species because *K. pintolopesii* and *K. heterogenica* had nearly identical sequences for this third gene (Kurtzman et al., 2005). From the preceding analyses, it appears that *K. heterogenica* is a hybrid between *K. pintolopesii* and an undescribed species of *Kazachstania* (Figure 4). This example raises the interesting question of whether apparent hybrids should be described as species. *K. heterogenica* is known from four strains, which were isolated from rodents in New Zealand, Portugal and the US. From analysis of three genes, the four strains examined appear genetically homogeneous and seem to qualify as a species distinct from other members of *Kazachstania*. Certain of the *Pichia* and *Starmera* species listed in Table 2 show disproportional substitution rates among the genes sequenced, thus raising the possibility that they may also be hybrids. These results do not resolve the question asked about describing hybrids as species, but raise the interesting possibility that interspecific hybridization may be a driving force in the formation of new species.

Is one strain enough?

From the foregoing examples, multigene comparisons provide a powerful means for resolving closely related species, including apparent hybrids and strains with gene polymorphisms, and it appears that new species can be accurately resolved whether based on a single strain or on multiple strains (e.g., Kurtzman and Robnett, 2003; Figure 4). Consequently, in addition to the commonly used D1/D2 and/or ITS, one or more protein coding gene sequences should be utilized for recognition of species.

The preceding examples address whether closely related species can be resolved from molecular comparisons, but other concerns about single-strain species descriptions remain. For example, the argument that intraspecific genetic diversity or species ecology is unknown from single strain descriptions is correct. However, when new species are described from multiple strains, the strains are often isolated from one geographical area and from the same substrate, thus providing little information on genetics or ecology because the population may be clonal. History has demonstrated that once a species is described, whether from a single strain or from multiple isolates, additional strains are often recognized from other substrates or geographical areas, thereby providing an incremental understanding of the species characteristics and distribution. For example, *Babjeviella* (*Pichia*) *inositovora* was described three decades ago (Golubev et al., 1981) based on a single isolate from a peat bog in Russia. Gene sequence analysis showed the species to be phyloge-

netically isolated (Kurtzman and Robnett, 1998; Kurtzman and Suzuki, 2010). Recently, two previously unrecognized isolates of *B. inositovora* were discovered in the ARS Culture Collection (NRRL), and these strains had been isolated from insect frass collected from trees in Quebec, Canada and Washington State, USA. All three strains gave identical reactions on standard yeast fermentation and growth tests, but finding these additional strains, despite the apparent absence of physiological differences, has broadened our perspective of the ecology of the species. Another example is *Wickerhamomyces* (*Pichia*) *bisporus*, which was described 90 years ago from a single strain isolated from an insect tunnel in a spruce tree growing in Austria. A second strain was isolated 50 years after the first, but this time the substrate was a human scalp. Interestingly, the second strain showed much slower fermentation of glucose and failed to grow on three carbon sources utilized by the first strain. Strain variation such as this is usually not published as a separate emendation of the species description, but is included in monographic treatments of the genera and species concerned.

Candida tanzawaensis (Nakase et al., 1988) is another example of not finding additional strains of a species in a timely manner. This species was described from a single strain 22 years ago and has not been isolated since. However, 25 species of *Candida* closely related to *C. tanzawaensis* have been described in recent years, thus considerably enlarging this initially isolated lineage (Kurtzman, 2001; Suh et al., 2004). For *Candida* and other asexual taxa, additional strain isolations may give complementary mating types if the species is heterothallic. *Saitoella complicata* represents another interesting example of describing species based on a limited number of isolates (Goto et al., 1987). The species, which phenotypically resembles the basidiomycete yeast *Rhodotorula*, is actually an ascomycete that is phylogenetically placed in a sparsely populated basal region of the Ascomycota, apparently near the branch-point with the Basidiomycota. The species is known from two strains isolated from soil collected in Laya, Bhutan. If this species had not been described because of concerns that not enough strains were available, this would represent a significant omission to our understanding of ascomycete phylogeny. The preceding examples illustrate the phylogenetic impact of describing single-strain species, and these examples tell us that for some species, little phenotypic variation is shown between the initial strain and subsequent isolates, whereas for other species, phenotypic and genetic properties would be notably underestimated in the absence of additional strains.

The following considerations are offered when confronted with description of a single-strain species. If phylogenetic analysis shows the species to be well separated from neighboring species or if physiological and genetic characterization demonstrate novel properties, the decision to describe the species is much easier than if the new taxon is scarcely resolved from members of a heavily populated clade. Another possibility is to deposit gene sequences of the new single-strain species in GenBank with the anticipation that once additional strains are discovered, a multi-strain species can be described, perhaps in collaboration with those who have found additional strains. For this possibility to be realized, the strain must be placed in longterm preservation and many culture collections are reluctant to accession material that may never be described.

In summary, multigene sequence comparisons have provided an accurate means for species identification. From the examples provided, it seems clear that there will be greater loss to our understanding of microbiology and biodiversity when single-strain species are not described than if the description had waited until additional strains could be included.

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Nomenclature of the Cyanobacteria/Cyanophyta – current problems and proposed solutions

Notes based on a roundtable discussion held on 16 August 2010 during the 18th Symposium of the International Association for Cyanophyte Research, České Budějovice, Czech Republic

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This paper presents a report on the roundtable discussion on nomenclature of Cyanobacteria/Cyanophyta that took place on 16 August 2010 during the 18th Symposium of the IAC in České Budějovice, Czech Republic. It highlights the current problems with the dual nomenclature system that have emerged as new taxa of Cyanobacteria/Cyanophyta can be named under the rules of the International Code of Botanical Nomenclature or the International Code of Nomenclature of Prokaryotes. The report presents updates about the current attempts to harmonize the nomenclature of the Cyanobacteria/Cyanophyta under the two Codes.

Introduction

Questions relating to the nomenclature of Cyanobacteria/Cyanophyta are regularly discussed during the triennial symposia of the International Association for Cyanophyte Research (IAC). Notes have been published based on discussions devoted to this topic at the 16th IAC Symposium held in Luxembourg in 2005 (Hoffmann, 2005; Compère, 2005; Oren and Tindall, 2005) and the 17th IAC Symposium held in Mérida, Mexico, in 2007 (Oren et al., 2010).

Another roundtable discussion took place on 16 August 2010 during the 18th Symposium of the IAC in České Budějovice, Czech Republic. The notes below are based on the discussions at that meeting.

The problem – a short history

The nomenclature of Cyanophyta/Cyanobacteria has traditionally been governed by the International Code of Botanical Nomenclature. Thus, Preamble 7 of ICBN lists blue-green algae among the "organisms traditionally treated as plants" to which rules and recommendations apply, and article 13.1 makes special provisions for the priority of names of certain groups of cyanobacteria: "Valid publication of names for plants of the different groups is treated as beginning at the following… *Nostocaceae Homocysteae*, 1 January 1892, *Nostocaceae Heterocysteae*, 1 January 1886" (McNeill et al., 2007).

Based on the realization that Cyanobacteria are prokaryotes, Stanier and colleagues published a proposal that the nomenclature of Cyanobacteria shall be governed by the provisions of the International Code of Nomenclature of Bacteria (ICNB; now ICNP) (Stanier et al., 1978). The proposal was discussed by the Judicial Commission of the ICSB in Munich in 1978. The Commission expressed the view that "scientists who believe blue-green algae to be bacteria should be at liberty to use the Bacteriological Code for their nomenclature" (Holt, 1979). A formal proposal was even made to recognize cyanobacterial names validly published under the provisions of the ICNB; at its meeting in Manchester

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Abbreviations: GCBN, General Committee on Botanical Nomenclature; IAC, International Association for Cyanophyte Research; ICBN, International Code of Botanical Nomenclature; ICNB, International Code of Nomenclature of Bacteria; ICNP, International Code of Nomenclature of Prokaryotes; ICSB, International Committee on Systematics of Bacteria; ICSP, International Committee of Systematics of Prokaryotes; IJSB, *International Journal of Systematic Bacteriology*; IJSEM, *International Journal of Systematic and Evolutionary Microbiology*.

in 1986, the Judicial Commission unanimously agreed to recommend to the ICSB that "those names of taxa of *Cyanobacteria* or *Cyanophyta* that are valid under the Botanical Code be considered valid under the Bacteriological Code for the purpose of preparing an acceptable list comparable to the Approved Lists of Bacterial Names" (Jones, 1987). However, no action was taken by the ICSB on this issue.

As a result a dual nomenclatural system has emerged in which new Cyanobacteria/Cyanophyta have been named either according to the provisions of the ICBN or the ICNB/ICNP. The problem associated with such a dual nomenclature system has led to extensive discussions, and several attempts were made to bridge the gap between the two approaches (Friedmann and Borowitzka, 1982; Castenholz and Waterbury, 1989; Whitton, 1992; Oren, 2004). For example, at a symposium on 'taxonomic concepts in "blue-green algae" – towards a compromise with the Bacteriological Code?', held in Sydney during the XIIIth International Botanical Congress in 1981, the following recommendations were issued (Friedmann and Borowitzka, 1982):

1. Blue-green algae (cyanobacteria) may be described following either the Botanical or the Bacteriological Code, with nomenclatural types chosen according to the Rules of each Code;

2. When describing a blue-green alga according to the Botanical Code, all efforts should be made to obtain a living pure culture which then should be deposited in a permanently established culture collection;

3. When describing a cyanobacterium according to the Bacteriological Code, a large sample of the type culture should be preserved (preferably as a dry specimen) which then should be deposited in a permanent institution (herbarium) and the description should be accompanied by photomicrographs or drawings;

4. Names of Cyanophyta validly published under the Bacteriological Code as Cyanobacteria are valid according to the Botanical Code;

5. Names of Cyanobacteria validly published under the Botanical Code as Cyanophyta are valid according to the Bacteriological Code.

These recommendations were never officially proposed to and accepted by the ICSB/ICSP or by the GCBN; however, item (4) was already covered by Art. 45(4) of the ICBN (see below), and recommendation 8B of Art. 8.4 of the ICBN is relevant to item 2.

A statement similar to the above item 5 was made at the meeting of the ICSB Subcommittee for the Taxonomy of Phototrophic Bacteria in 1985 in Paris, proposing that "names of cyanobacteria described and validly published as blue-green algae under the ICBN are recognized as having been validly published under the ICNB" (Trüper, 1986).

In the past decade questions relating to the nomenclature of Cyanobacteria/Cyanophyta have been discussed in different frameworks, including the ICSP (Labeda and Oren, 2008), the ICSP Subcommittee on the Taxonomy of Photosynthetic Prokaryotes (Madigan and Imhoff, 2007), and others (Oren, 2004). The triennial IAC symposia where the cyanobacteria taxonomists meet proved an excellent opportunity to discuss issues of nomenclature (Hoffmann, 2005; Compère, 2005; Oren and Tindall, 2005; Oren et al., 2010). We here summarize some of the topics discussed at the 18th IAC symposium held in České Budějovice in August 2010.

Are the cyanobacteria formally part of the Bacteriological Code?

General Consideration 5 as published in the 1990 revision of the ICNB (Lapage et al., 1992) simply stated that "This *Code of Nomenclature of Bacteria* applies to all bacteria. The word "Cyanobacteria" was not mentioned in the Code. This is not surprising as the ICNB/ICNP does not make special provisions for individual groups of prokaryotes, this in contrast to the ICBN which contains special articles, statements and exceptions, for many taxonomic groups covered that are governed by the Code [e.g., special starting dates for valid publication of two groups of algae (Cyanobacteria) as given in Article 13.1.(e)]. The fact that the Cyanobacteria were not explicitly stated in the ICNB/ICNP did therefore not exclude them.

A proposal to update and make changes to the ICNB by Tindall (1999) included a proposal to update General Consideration 5 as follows: "The term "bacteria" covers those organisms that are variously recognized as prokaryotes, *Bacteria*, *Archaea*, *Eubacteria* and *Archaeobacteria*. Due consideration has been given to include cyanobacteria, which are traditionally covered by the International Code of Botanical Nomenclature, and has been discussed elsewhere". However, the version of the Note to General Consideration 5 approved by the Judicial Commission and endorsed by the ICSP at their meetings in Sydney in 1999 was very different: '"Prokaryotes" covers those organisms that are variously recognized as e.g., *Schizomycetes*, …, *Schizophycetes*, *Cyanophyceae* and *Cyanobacteria*" (De Vos and Trüper, 2000; Labeda, 2000). The additional information that "Due consideration has been given to include cyanobacteria …"'

was not included in the Code. The proper meaning of this sentence can be interpreted in different ways.

Very few names of cyanobacterial species have thus far been validly published based on the rules of ICNP. Notable examples are *Halospirulina tapeticola* (Nübel et al., 2000), *Planktotricoides raciborskii* (Suda et al., 2002), and the recently described *Rubidibacter lacunae* (Choi et al., 2008). Also the names of the family *Prochlorotrichaceae*, the genus *Prochlorothrix*, and the species *Prochlorothrix hollandica* (Burger-Wiersma et al., 1989) have standing in the nomenclature under the rules of the ICNP. Some other names proposed under the rules of the ICNP are illegitimate because of technical errors (Oren, 2004; Oren and Tindall, 2005; see http://www.bacterio.cict.fr/classifcyano.html for more information). The ICNP is not independent of the ICBN (Principle 2), and therefore it is formally impossible to validly publish the name of a new species under the rules of the ICNP for a genus previously named under the provisions of the ICBN. The proposal of *Tychonema bourrellyi* (Suda et al., 2002) is an example. Likewise, *Microcystis aeruginosa* (Otsuka et al., 2001) is an illegitimate name under the provisions of the ICNP as the name of the genus was never validated. The names *Prochlorales*, *Prochloraceae*, *Prochloron* and *Prochloron didemni* are problematic, in spite of the attempts made to validly publish these names under both Codes (Florenzano et al., 1986; Hoffmann and Greuter 1993). At present there is no clear answer as to which authorities and dates should be ascribed to this name. Another problematic name is *Prochlorococcus marinus* subsp. *pastoris*, proposed by Rippka et al. (2000) (for details see Oren and Tindall, 2005).

Bergey's Manual of Systematic Bacteriology is a handbook widely used by bacteriologists. The last edition classifies the Cyanobacteria in "form genera" that correspond with names validly published under the ICBN (Castenholz, 2001). The term "form genus" was introduced based on observations that a single form with very characteristic morphology often dominates cyanobacterial populations, and that strains with such morphologies often have global distributions and can readily be identified in diverse geographical localities. All the names of form-genera and all cited species are derived from the botanical nomenclature, but not respecting the original type species. *Bergey's Manual* is not an official publication of the ICSP, and publication of new names in the manual does not provide them the status of validly published names. Accordingly, the names of these "form genera" have no standing under the ICBN or under the ICSP.

When naming Cyanobacteria, the ICBN and the ICNP are mutually incompatible

The dual nomenclature system for Cyanobacteria/Cyanophyta is as problematic as the two Codes differ in many essential features. A short overview of the relevant points where the codes differ is sufficient here, as the topic has been extensively reviewed by the past (Compère, 2005; Hoffmann, 2005; Oren, 2004; Oren and Tindall, 2005; Oren et al., 2010).

1. Botanical nomenclature is independent of zoological and bacteriological nomenclature (ICBN Principle 1), but the nomenclature of Prokaryotes is not independent of botanical and zoological nomenclature (ICNP Principle 2). Therefore it is impossible to validly publish the name of a new species under the rules of the ICNP for a genus previously named under the provisions of the ICBN (see above).

2. Priority of names: Valid publication of names for plants of the different groups is treated as beginning at … : ALGAE, 1 May 1753 … Exceptions: *Nostocaceae Homocysteae*, 1 January 1892, *Nostocaceae Heterocysteae*, 1 January 1886 (ICBN Art. 13.1). Under the ICNP, priority of publication dates from 1 January 1980 (ICNP Rule 24a). With the publication of the "Approved Lists of Bacterial Names" (Skerman et al., 1980) a new starting was created in the nomenclature of prokaryotes. These lists did not include any names of Cyanobacteria.

3. Frameworks for the valid publication of new names of taxa: Under the Botanical Code, restrictions do not exist on the journal in which new names may be validly published. Publication is effected … only by distribution of printed matter … to the general public or at least to botanical institutions with libraries accessible to botanists generally (ICBN Art. 29.1). As a result, botanical nomenclature information is widely scattered in the literature, and there is no central registration/indexing. Under the provisions of the ICNP, there is one single journal where new names should be published to obtain standing in the nomenclature, and that is IJSB/IJSEM, either as original articles or by inclusion in the "Validation Lists" ("Validation of the publication of new names and new combinations previously effectively published outside the IJSB/IJSEM") published periodically in that journal (ICNP Rules 24a, 24b). The date of publication is that of publication in the IJSEM. The name may be mentioned in a previously published description ("effective publication"), but the name is not validly published until its publication in the IJSEM (Rule 27); only names

that conform to the Code may be validly published. The new starting data of January 1980 with the publication of the "Approved Lists", together with the central registration/indexing of names validly published, provide a simple means of keeping track of the validly published names (= indexed names that conform to the Code). The website www.bacterio.cict.fr, established and faithfully maintained by Jean Euzéby (Euzéby, 1997) provides updated and reliable information on validly published names of prokaryotes.

4. The nature of the nomenclatural type. For plants, type specimens of names of taxa must be preserved permanently and may not be living plants or cultures. Instead they are non-viable specimens preserved in herbaria, or illustrations. However, cultures of fungi and algae (including Cyanophyta) preserved in a metabolically inactive state (e.g., by lyophilization or deep-freezing) are acceptable as types (ICBN Art. 8.4). On the other hand, under the Bacteriological Code for each newly described species a living type strain should be designated and subcultures must be made available from at least two publicly accessible service collections in different countries (ICNP Rule 30).

What should be done to harmonize the treatment of the Cyanobacteria under both codes?

In the publications summarizing roundtable discussions during the previous two IAC symposia (Oren and Tindall, 2005; Oren et al., 2009), ideas were presented about how it could be possible in the future to harmonize the nomenclature treatment of the Cyanobacteria under the two Codes. Appropriate changes will have to be made in each Code, and these include special provisions for the Cyanobacteria nomenclature in the ICNP, a document that does not contain any specific Rules for any specific group of prokaryotes. Basically the following will be needed:

1. Central registration and indexing of names should be accepted for the Cyanophyta/Cyanobacteria under the ICBN.

2. The ICNP should reciprocate Article 45.4 of the ICBN that states that "if the taxon is treated as belonging to the algae, any of its names need satisfy only the requirements of the pertinent non-botanical *Code* for status equivalent to valid publication under the present *Code*". Therefore, the name of a cyanobacterium validly published under the ICNP is automatically considered valid under the ICBN, provided it is not a homonym of another plant taxon. Bacteriologists have earlier recommended reciprocating Article 45.4 of the ICBN (Jones, 1987); however, this recommendation was not implemented.

3. A "List of Approved Names" should be drawn up to make a new start in the nomenclature of the Cyanobacteria, and this list should provide information about the nomenclatural type of each species, whether based on the Articles of the ICBN or the Rules of the ICNP.

The Special Committee on the Nomenclature of Cyanophyta/Cyanobacteria

During the XVII International Botanical Congress (Vienna 2005), the Nomenclature Section resolved "to establish a Special Committee on the Nomenclature of Cyanophyta/Cyanobacteria, in association with relevant appointees from the Commission on Prokaryote Nomenclature, to report to the next Congress" (McNeill et al., 2005), as proposed by the GCBN Committee for Algae (Compère, 2007). This Special Committee should consist of experts on the two Codes and official representatives of the GCBN and the ICSP. It should draft the necessary changes in the Codes by which a name may be considered to be validly published, in cooperation with the appropriate botanical and bacteriological authorities, and prepare an "Approved Lists of Names" document, leading to a consensus nomenclature.

Nominations for membership in this Special Committee were made at the 2006 meeting of the ICSP Subcommittee on Phototrophic Prokaryotes, held in Pau, as follows: L. Hoffmann (chairman), A. Oren (secretary), P. Compère, V. Demoulin, S. Golubic, T. Joosten, J. Komárek, W. Prud'homme van Reine, B.J. Tindall, H.G. Trüper, S. Ventura, and A. Wilmotte, with F.R. Barrie and J. McNeill as ex-officio, non-voting members (Madigan and Imhoff, 2007). These nominations were submitted by L. Hoffmann to the GCBN in December 2006. In spite of numerous reminders the GCBN has never responded. At the time of writing (August 2010) formal approval was still pending, and the proposed Special Committee has never convened and has not started its work.

To strengthen the interactions of the botanical experts with the authorities responsible for the ICNP, the ICSP at its meetings in San Francisco in 2005 co-opted L. Hoffmann as a member to participate in the meetings of its Judicial Commission. Dr Hoffmann took part in the discussions during the meetings of the ICSP Judicial Commission in Istanbul in 2008.

Preparation of Approved Lists of Names of Cyanobacteria/Cyanophyta

A new start in the Cyanobacteria/Cyanophyta nomenclature under both Codes should begin with the publication of 'Approved Lists of Names", comparable with the "Approved Lists of Bacterial Names" (Skerman et al., 1980). Such a list would not necessarily replace the priority dates of 1 January 1892 (*Nostocaceae Homocysteae*), 1 January 1886 (*Nostocaceae Heterocysteae*), and 1 May 1753 (all other groups), as currently regulated by the ICBN. All names on the list will be protected names validly published under both Codes that cannot be replaced by earlier (or other) synonyms not included on that list.

A special problem is the lack of proper types for many of the names of cyanobacterial species names in current use. Each name on the "Approved Lists" to be established should have an available type specimen (ICBN) or type strain (ICNP) that should fulfil the requirements of the respective Codes. If necessary, epitypes should be designated to replace missing or incompletely described type specimens.

A list of generic names of Cyanophyceae/Cyanobacteria in "common use" was presented by L. Hoffmann to be discussed at the 17th IAC Symposium, Mérida, Mexico, 2007. It was prepared mainly on the basis of the NCU-3 list: names in current use for extant plant genera, compiled and edited by Greuter et al. (1993). This incomplete list, from which some names should be removed as insufficiently known, illegitimate, or belonging to groups other than cyanobacteria, encompassed 230 genera, with in addition 5 validly published names of genera based on the Rules of the ICNP, 11 non-valid names in common use or recently published, and 222 "other names', which contain mostly synonyms or names of taxa eliminated from cyanobacteria. The preparation of "Approved Lists of Names of Cyanobacteria/Cyanophyta" will therefore be an excellent opportunity to re-evaluate the existing names of taxa. Many are no longer in use, and for many it is unclear whether they are indeed validly published. Synonyms abound, and for many species the nomenclatural type is no longer available.

An updated version of the lists of genera of Cyanophyta, as prepared by J. Komárek and T. Hauer is now available online at www.cyanodb.cz, and this version can be used as the basis for the preparation of the "Approved Lists", first of genera, then also of species. Information on the nomenclatural types is included in these lists. The list of genera and the corresponding database (information about species) are continually updated. The discussions in Mérida – 2007 and České Budějovice – 2010 called upon all experts to contribute to the completion of these lists. Species to be included in the future should be fully documented with reference to the type material, the Latin diagnosis (if based on a description under the ICBN), and the nomenclatural history. This will also be the opportunity to correct any grammatical and orthography problems that may be associated with the currently used names. After publication of the "Approved Lists", names of newly described taxa must be validated before they will obtain standing in the nomenclature, based on a procedure comparable to that currently in use for other prokaryote taxa (Tindall et al., 2006).

Progress with the compilation of the lists has been relatively slow. Following discussions at the 2009 meeting of the ICSP Subcommittee on the Taxonomy of Photosynthetic Prokaryotes in Vancouver, it was decided to invite all experts who could contribute to the preparation of the lists to a meeting in Kiel in the beginning of 2010. However, except for the two authors of this essay nobody replied to the invitation. In view of this highly disappointing turnout the meeting was canceled. Further progress should be made by electronic communication, and the www.cyanodb.cz web site can be an excellent framework for that purpose.

The current lists include a list of 14 non-validly published names in common use. Among these are *Acaryochloris*, *Chlorogloeocystis*, *Crocosphaera*, *Cyanobacterium*, *Cyanobium*, *Cyanospira*, *Euhalothece*, *Gloeobacter*, *Halomicronema*, *Halothece*, and *Thermosynechococcus*. Attempts have been made or are under way to solve the problem for two of these genera: *Halothece* and *Gloeobacter*.

The paper by Margheri et al. (2008) was intended to comprise "the valid description of the genus *Halothece* based on the type strain MPU 96P605", and it provided formal descriptions of *Halothece* gen. nov. and *Halothece californica* sp. nov. under the ICBN, so that now the genus can be deleted from the list of non-validly published names. Additional information toward future validation of the names under the rules of the ICNP was provided. However, validation of the names under the rules of the ICNP is technically not possible (Oren, 2009). In the case of the genus *Gloeobacter* and the species *Gloeobacter violaceus* as described by Rippka et al. (1974), these names have no standing under the ICNP as the names did not appear in the Approved Lists of Bacterial Names of 1980 (Skerman et al., 1980) or in later validation lists. The names also have no standing under the ICBN as the type is a living culture and no Latin description or diagnosis was ever given. Theoretically, validating the names under the Rules of the ICNP should not be problematic based on PCC 7421 = ATCC 29082 as type strain, isolated in Kastanienbaum, Switzerland in 1972. However, it is well possible

that *Gloeobacter violaceus* may be identical to *Gloeothece coerulea* and *Aphanothece caldariorum* (Mareš, 2010), and the nomenclatural status of the organism will therefore need a more in-depth evaluation. A formal proposal to validate the names *Gloeobacter* and *Gloeobacter violaceus* under the rules of the ICNP has recently been submitted to IJSEM (Mareš, J., M. Gugger, R. Rippka, J. Komárek and A. Oren. Proposal for the validation of the names *Gloeobacter* (*ex* Rippka *et al.* 1974) gen. nov. nom. rev. and *Gloeobacter violaceus* (*ex* Rippka *et al.* 1974) sp. nov., nom. rev.).

The "Cyano-Guide" and its status

During the 8th IAC symposium held in Kastanienbaum in 1979, a proposal was made to establish a special document to guide scientists who wish to describe and name new taxa of cyanobacteria, based on the concepts given in the ICBN and the ICNP. Over the years this "Cyano-guide" (Guide to the nomenclature treatment of oxyphototrophic prokaryotes (Cyanoprokarotes and Chloroprokaryotes) (Komárek and Golubić, posted online at www.cyanodb.cz), has evolved into a long document, consisting of General Considerations, Articles, and Recommendations in the style of the two Codes. Although not formally presented as a special "Cyano-Code" to replace (now or in the future) the ICBN and the ICNP as the document that should regulate cyanobacterial nomenclature, *de facto* the document resembles a Code. It was the intention of the authors that the guide should as much as possible correspond with demands of the Bacteriological and Botanical Codes.

It cannot be expected that the authorities of the ICBN – the GCBN and the plenary session of the International Botanical Congress – will ever agree to transfer the authority that governs cyanobacterial nomenclature to a separate Code. However, as a document to guide scientists during the preparation of descriptions of cyanobacteria, based on laboratory strains as well as material collected from nature, the "Cyano-Guide" will be extremely useful. Treatment of the topic is polyphasic, and completely conforms to the current practice in bacteriology (Tindall et al., 2010) and pioneered for the cyanobacteria by Stanier and his coworkers in the 1970s (Stanier et al., 1971; Rippka et al., 1979). It includes morphological description (taking into account the fact that many species show considerable morphological variability when growing under different environmental conditions), 16S rRNA gene sequencing to provide phylogenetic information because correspondence between 16S rRNA-based phylogeny and morphology is often poor (Wilmotte, 1994; Wilmotte and Herdman, 2001), DNA–DNA hybridization experiments (Wayne et al., 1987; Stackebrandt et al., 2002), introduced in cyanobacterial studies already in the 1970s (Stam and Venema, 1977; Stam, 1980), and information on many other properties to be tested to make characterization of cyanobacteria and the description of new species as complete as possible.

Thus, the "Cyano-Guide" is an extremely helpful document "for internal use", but unless formal approval is obtained from the authorities responsible for the ICBN and the ICNP, the "Cyano-Guide" cannot be used as a "Cyano-Code" to replace one of the existing Codes.

Final comments

The summary statement of the roundtable discussion in Mérida in 2007 (Oren et al., 2010) gave an optimistic vision. The intention was to have soon thereafter a functioning "Special Committee" to discuss the details needed for harmonization of the nomenclature of the Cyanobacteria under both Codes, and to complete a proposed "Approved Lists" document to the species level within a short time. The "Special Committee" still has not been formally approved, and the "Approved Lists" document is still no more than a rough draft on the genus level only.

The "dream scenario" that ends the roundtable summary from Mérida stated 2011 as the year in which all problems could be solved, a year in which both the Botanical Congress and the ICSP and its Judicial Commission will convene. This opportunity has been lost now, and the next similar opportunity will probably not before 2017. In summary, some progress has been made, but it still will take much time before the final goal will be achieved when we will have one, simple, transparent, and universally accepted nomenclature for the Cyanobacteria/Cyanophyta.

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Reminiscences and reflections

Don J. Brenner

I declined when first asked to contribute an autobiographical sketch to *The Bulletin*. I thought that a biography would be more accurate and certainly more objective than an autobiography, and I wondered if my contributions merited the time they would take to recount. Then I decided that it would be nostalgic and self-illuminating to reminisce about the people and events that shaped my life. These are events as I remember them. I apologize in advance to anyone I inadvertently forgot and for any inaccuracies.

Life began on 1 November 1936 in Astoria, New York. I was the first son of Olga and Charles Brenner. Three years later, my brother Bernard was born prematurely, weighing 3 pounds 15 ounces. He became immediately famous as the only boy among 50 premature babies in incubators at the 1939 World's Fair in Flushing Meadow (now the home of the US Open tennis tournament). That incubator saved his life. Going to that World's Fair is my earliest memory.

My parents divorced in 1943. During our childhood, my mother worked at a number of jobs, including making decorative aprons and teaching Latin dancing, but mainly selling advertising for Women's Wear Daily. Bernie and I were what are now called latch-key kids, fending for ourselves after school (and that was before television). As a boy, I played most conventional sports–softball, baseball, basketball, football, and my mother taught me how to play tennis. In New York City we also played roller derby, roller hockey, stick ball, stoop ball, box ball, slap ball, punch ball, regular and Chinese handball, mumblepeg, ring-a-leevio, and flipping baseball cards. Unfortunately, I also developed some bad habits such as pitching pennies and smoking.

I was a good student in elementary school, mainly because my mother taught me how to read and to do arithmetic before I started school (Figure 1). The public school (grades 1–6) was a block from our home. I usually walked to the junior high school that was 1½ miles away. It was in a mixed neighborhood with some gang activity. A student was shot to death at one of the dances and there were a couple of incidents where students attacked teachers. I was an indifferent student, but did start singing and radio acting. William Cullen Bryant High School was just over 2 miles away and I either walked or commuted by bus. Here too I was an indifferent student, but began to be intrigued by biology. I continued with radio theatre and played varsity tennis. I also attended NBC TV singing school, which was the catapult to "TV Teentimers Club", a popular TV talent show for teenagers. That ended when my voice changed.

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Figure 1. Near the beginning: Don, Olga and Bernie Brenner in the mid-1940s.

I applied to Hunter College and St Johns University, both in New York City, and to Marietta College. The reply from St Johns stated that I was not qualified for science and that I should consider another field – perhaps they knew something? I was accepted at Hunter and Marietta and decided to go to Marietta, a small liberal arts college in Marietta, Ohio. The deciding factors, in addition to their strong biological sciences program, were going out of town to a campus and going to a small school (my high school had 4000 students; Marietta had 744). Since I had skipped a year in junior high school, I headed for Marietta in 1953, 2 months before my 17th birthday. It should be noted that tuition, room and board for a semester totaled about $450! I received $5/week for spending.

Marietta had a faculty of three in the biology department. Two of them had a profound influence on my future career.

Dr Paul Seyler, who taught zoology and comparative anatomy, was a stickler for accuracy and detail as well as for the theory behind the facts. He made you think on your feet by having one-on-one practical exams in which he literally threw a bone (or other organ or part) at you and peppered you with questions. He greatly impressed (and sometimes frightened) me and helped develop in me a logical scientific approach and an appreciation for accuracy. Professor Harla Eggleston taught histology, embryology, bacteriology and special problems courses. He was a kindly gentleman with glasses and an ever-present pipe (this was before prohibitions on smoking). If you can imagine a taller, thinner version of the character actor Barry Fitzgerald (for those of you who are old enough), you have a picture of Professor Eggleston. He introduced me to bacteria and to evolution, the two areas that would become the basis for all of my future research. Professor Eggleston was passionate about biology. In his gentle way, he infused most of the biology majors with his passion and respect. I might add that somehow I (and everyone else) made it through college and graduate school without the aid of a computer or a cell phone (or a credit card).

While in college I worked every summer – mailroom, two factories, and tennis counselor. As I think back about jobs, I realize how lucky I was in terms of graduate education and job choices. I was accepted by every place I applied to for graduate and postdoctoral studies. After my first job I never looked for employment. The two positions that comprised my career dropped into my lap without any effort on my part.

I returned to New York after graduation from Marietta College in 1957 with a B.S. degree in biology. I was accepted at Long Island University (Brooklyn, NY) for graduate work in biology. I took a full-time job at the Sloan-Kettering Institute for Cancer Research at one of their laboratories in Brooklyn that was walking distance from Long Island University. I would work until 5: 00 and then walk about a mile to the University, eat dinner, go to class from 7: 00 to 10: 00 or 10: 30 (4 days a week) and arrive home close to midnight. Then the Sloan-Kettering laboratory moved to Rye, NY, which is about 30 miles from the University. I followed the same routine, except there was no time for dinner until I got home. At Sloan-Kettering I worked under Dr Christine Riley, who was a student of Dr Selman Waxman at Rutgers University when he won the Nobel Prize. Dr Riley ran an anti-tumor drug screening laboratory during the period when the irrational approach to anti-cancer drugs was in vogue (test everything and something might work). The test system was Sarcoma 180 in mice. It was repetitive and uninspiring work (injecting mice, administering the test drug and measuring the tumor response), but Dr Riley taught me about sterile technique and scientific record keeping. She must have done something right, because five of the young technicians in her laboratory went on to doctoral degrees in medicine or microbiology. For convenience, I chose to do my MS thesis at Sloan-Kettering on the effect of essential oils on Sarcoma 180 in mice. Not exciting, but time-wise it would have been next to impossible to do a thesis at the University. A number of the essential oils were effective in preventing tumor growth, but they were quite toxic and the toxicity could not be diluted out without eliminating their efficacy. I received my MS degree in biology in 1960.

After applying for predoctoral studies, I transferred to another Sloan-Kettering laboratory in Manhattan, which was much closer to my home. It was the tobacco carcinogenesis laboratory headed by Dr Ernest Wynder (he also ran the first private cancer clinic in the US). Dr Wynder was quite active socially, dating, among many others, Kim Novak, a Miss Universe, and many models. My experience in his laboratory taught me the value of a team approach to research.

I applied to the Microbiology Department in the Medical School at the University of Washington (Seattle) for PhD studies. They offered me a fellowship, which I immediately accepted. A sign of the times is that the $2000 stipend was enough to live on (barely). Another is that the turbojet flight from Seattle to N.Y. took 10½ hours (unless there was fog between Seattle and Spokane, which was often the case) with stops in Spokane, three stops in cities in Montana, and Chicago or St Louis. On more than one occasion, they asked passengers if they would leave the flight so they could fill their seats with mailbags full of Christmas cards.

Shortly after their arrival, the Microbiology Department holds a meeting with new students to assess their strengths and weaknesses and to determine their course schedule. One of the first questions they asked me was whether I had any courses in medical microbiology. My reply, which I will never forget and always regret was: “No. I’ve avoided it like the plague.” When they had finished questioning me, they sent me outside and deliberated. Dr Howard Douglas came out, put his arm around my shoulder and told me that the first courses I would take were three quarters of medical microbiology.

The Microbiology Department had a deservedly splendid reputation. It had wonderful laboratory instructors, excellent post-doctoral fellows, and was filled with top-notch faculty members. Two of the lab instructors merit particular mention. Ramona Memmer is a gentle, yet unabashed liberal, antiwar person. Now retired, she was a dedicated, patient

teacher, regardless of whether the class had nursing, dental, medical, or graduate students. She taught me many things about teaching, patience, and the laboratory (and politics). Dr Richard (Dick) Levin is unique in every way. His first love was music. He has a M.A. in music and a PhD in microbiology. Everyone loved Dick. He was infectiously enthusiastic about everything. He spoke so earnestly and listened so hard that you seemed to be the most important person on earth. Dick taught the nursing course. He gave a review the night before the final. One year there was a storm with more than 10 inches of snow. Everything on campus stopped, but almost all of the nursing students made it to the review – a combination of love and fear. When Dick left the University to go to Oberlin College he was given an off campus party. Four carloads of nursing students attended. I learned a great deal about teaching and humanity (and folk singing) from Dick.

Two of the postdoctoral fellows were good friends and role models. Dr John Johnson was one of the first to use DNA hybridization for bacterial classification. He went on to a brilliant career at Virginia Tech before being tragically struck down by Lou Gehrig's disease. I tried to emulate John's innovative, yet careful approach to research, work ethic, and objectivity. Dr DeLill Nasser, also died prematurely. She endured much more than her share of hardship and inequity, but never complained. I prized her friendship and greatly admired her ability to solve problems or function despite them.

Almost all of the Microbiology Department faculty made an impression on my career, but Drs Neal Groman and John Holland molded my PhD studies and subsequent career. I asked to work under Dr Groman because I wanted to work on bacteriophages and he was an expert on lambda bacteriophages and on diphtheria toxin (a phage-mediated trait). Dr Groman's mentoring style was to allow you to make mistakes and by discussing them, force you to work your way through them. His avocations were writing poetry and playing the recorder. He was a marvelous lecturer (the medical students rated him as their best lecturer). His wisdom and patience reminded me of a Talmudic scholar.

My thesis title was: "The inhibition of coliphage reproduction following super infection of induced lysogens". It was a modest project with acceptable results.

There were two memorable incidents during the course of the work. There are two types of bacteriophage – virulent and lysogenic. Virulent phage infect bacteria, reproduce and lyse the bacterial cells producing a clear plaque in a lawn of host bacteria. Lysogenic phage infect bacteria and can reside within their host cells until induced (usually by ultraviolet light). After induction they reproduce and lyse the host cells, producing a cloudy plaque. To determine inhibition of lysis, one counts the decrease in plaques on the inhibited plates, compared to the uninhibited, control plates. Late one night I had to count plaques on several hundred plates. I did it without a break, 4–5 hours. When I tried to stand up, I collapsed to the floor in pain from severe back spasms. I lay there for some 20 minutes before I was able to painfully stand and limp home. That was the start of the chronic back problems I have had ever since.

One of the better-known research groups dealing with lysogenic bacteriophage had published on a mutant that I requested for my research. They sent a bacterial culture that was supposed to harbor the mutant. They either sent the wrong culture, or the culture was contaminated with a T-phage, which is virulent and spells disaster for any laboratory working with lysogenic phage. Every culture had to be discarded and the laboratory thoroughly disinfected. It cost me 2 months and painfully taught me a valuable lesson – always check and authenticate cultures.

I have two vivid remembrances of my oral exam. Jacob, Monod and their colleagues at the Institut Pasteur had begun to publish their studies on bacterial genetics. I followed their work closely and fortunately so did some of the panel. I did well on their questions. One question that I did not answer to the committee's satisfaction was: "What is the most significant invention in the history of science?" I gave dozens of answers ranging from the sophisticated (electron microscope) to the sublime (Petri dish). The desired answer was the mechanical balance.

Dr Groman was pleasant and accessible, but always professional. After passing the oral exam and knowing (barring catastrophe) that I would obtain my PhD, he became extremely friendly. He told me that he once was very friendly to a student and then had to tell him that he would not receive his degree. Since that time he was purposely reserved until a student's degree was assured.

Dr John Holland was the youngest member of the Microbiology faculty. He was a brilliant virologist, having won the Eli Lilly Award for the best scientist under the age of 35. John was a favorite of all the graduate students for at least two reasons. He played three intramural sports with the graduate students and his office was always open. He had the rare ability to talk with you on any subject and for any length of time without interrupting his experiments.

When I had decided to do postdoctoral studies, I wanted to go to the Carnegie Institute of Washington where Dr Brian

McCarthy and other staff members were pioneering in studies using DNA hybridization to compare the genomes of bacteria. Then Dr McCarthy took a faculty position at the University of Washington and, since I wanted to go somewhere else for postdoctoral studies, I had to change my plans.

Having no plan B, I asked Dr Holland if he had any suggestions. He said that he had a good friend at the National Institutes of Health (NIH), Dr Bill Hoyer, who would be visiting soon.

Dr Hoyer and his colleagues were also using DNA hybridization in a variety of bacterial and viral systems, and had close collaborations at the Carnegie Institution of Washington. I spoke with Dr Hoyer and was very impressed with him and his program. He accepted me and I applied for and received a NIH Postdoctoral Fellowship (the princely amount of $5000 for the first year).

Bill had two MDs in his laboratory. David Axelrod, who later became the Commissioner of Health of New York State, and Mal Martin, who became a Division Director at NIH. They were all superb researchers with wonderful imaginations. Each had one or more bread and butter projects as well as a long-shot project. It was here that I learned about DNA hybridization, using the traditional DNA agar approach. They showed me how to have fun in the laboratory without compromising your work. I couldn't wait to get to the laboratory each morning and usually stayed into the night, wondering where the time went. My first experiments were with enterobacteria, a focus that stayed with me throughout my career. After the investment in training me and seeing the first signs of independent research progress, Bill did something that very few mentors would. He allowed and encouraged me to spend most of the second year at the Carnegie Institution of Washington.

The Terrestrial Magnetism Laboratory of the Carnegie Institution of Washington was world-renowned for its many achievements, most recently in the molecular biology of nucleic acids. Its high-powered staff included Drs Dick Roberts, Roy Britten, Dean Cowie, and Ellis Bolton. Dr Bolton and Dr McCarthy (who had recently left for the University of Washington–see above) had done many of the pioneering studies on DNA and RNA hybridization, on the methodology of measuring nucleic acid sequence similarities, and on the parameters that affect the hybridization reaction. Also at Carnegie, in his second year of postdoctoral work, was Dr Dave Kohne who did much of the early work on rRNA sequencing. He went on to become a staff member and eventually founded a company that identified bacteria and other micro-organisms on the basis of their rRNA similarity.

I worked under Dean Cowie, who was studying temperate bacteriophage relatedness. We published three papers on bacteriophage and bacterial DNA relatedness and on the effect of incubation temperature on the extent and stability of DNA duplexes. I only collaborated once with Dave Kohne, but we spoke continually and he had a substantial influence on my work during my stay at the Carnegie Institution and subsequently at the Walter Reed Army Institute of Research and at The Centers for Disease Control and Prevention (CDC).

The foregoing covers my childhood, education, and the people who influenced my scientific development. The remaining sections will cover the two institutions I worked at during my career, my collaborators, research accomplishments, non-research contributions, and regrets.

Walter Reed Army Institute of Research, 1967–1974

Howard Novitch and his wife Beryl were fellow technicians at Sloan-Kettering. Howard took a job with the New England Nuclear Corporation. He was headquartered in Maryland and we resumed our friendship. He told me that a job would be available in the Division of Biological Chemistry at the Walter Reed Army Institute of Research in Washington, D.C., just a few miles from NIH and the Carnegie Institution of Washington. They offered me the job, which I accepted on the condition that I would be allowed to develop a research program in bacterial nucleic acid relatedness. They agreed and I went to Walter Reed and started the DNA Hybridization Laboratory in 1967. I remained at Walter Reed until September of 1974.

Four people were instrumental in assuring the productivity of the DNA Hybridization Laboratory. Dr B.P. Doctor (Doc) was my supervisor (Figure 2). He allowed me complete freedom to design and carry out a research program and, as I only later fully appreciated, he shielded me almost completely from administrative duties. Dr Stanley Falkow left Walter Reed before I arrived. He went to Georgetown University and had close ties to the Carnegie Institution of Washington, where we met. Stan's contributions to the genetic basis of virulence and myriad aspects of medical microbiology border on legendary. I was fortunate to collaborate with him and many members of his group throughout my years at Walter Reed (Figure 3). Dick Fanning was a research technician at Walter Reed when I arrived. He rapidly learned the new techniques and he ran my laboratory, not only until I left Walter Reed, but thanks to the largesse of Doc, Dick spent most of his time on collaborative projects with me until he retired many years later. Arnie Steigerwalt

Figure 2. Going over data with Dr B.P. Doctor at Walter Reed in the 1960s.

Figure 3. A brief bearded period at Walter Reed during the early 1970s.

was in the US Army when he was assigned to my laboratory in 1971. When I moved to the CDC in September 1974, Arnie came with me. He and his wife and I became close friends, and I am Godfather to each of his three children. Arnie assumed responsibility for all aspects of the laboratory, including training visiting scientists, and remained in that capacity when I retired in 2000, until his retirement late in 2010.

In 1972, Stan Falkow asked me if I had ever met Dr William (Bill) Ewing of the CDC, who was the acknowledged "father of *Enterobacteriaceae*". Despite the fact that most of my research involved classification of *Enterobacteriaceae*, much of it in collaboration with Stan's group, I had never met Dr Ewing, even at a meeting. Stan was going to visit CDC and I went with him. After a few minutes, Stan left Bill and I alone. We sat on a couch in his office, lit our cigarettes (still legal) and began a friendship and collaboration that lasted until Bill's death. Bill taught me more than I thought possible about bacterial classification, both the laboratory aspects and the political realities. He loved it when the molecular methods proved him to be correct (which was most of the time), and accepted it on the infrequent occasions when they proved him to have been wrong. At the end of our first meeting, Bill told me that one day I would be at the CDC. At the time I gave no thought to this prophetic statement.

The Centers for Disease Control and Prevention, 1974–2000

Early in 1974, I received a phone call from Dr Vulus (Bud) Dowell, who was Chief of the Bacteriology Branch and the Anaerobic Bacteriology Section at CDC. I did not know him and was shocked when he invited me to interview for a position as Chief of the Enteric Section at CDC. I remember telling him that he must have the wrong person since I was not a clinical microbiologist, but he assured me that he wanted to institute a nucleic acid-based program, so I visited. He agreed to my requests, including bringing Arnie Steigerwalt with me, and the rest, as they say, is history. Bud kept every promise. He gave me complete autonomy and, as had Dr Doctor, shielded me from as many administrative duties as he could.

There was no place to do radioactive isotope work in the Enteric Section laboratories, so we had to take over a double laboratory and create one. As she was removing her cultures and equipment from the laboratory we were going to use, I first saw Frances Hickman, who would be a collaborator for some 35 years and my wife for 29 years and counting. Frances is one of the world's experts in many areas of enteric bacteriology, especially *Salmonella* and *Vibrio*. It was extremely fortunate to inherit many of the technicians, including Betty Davis, Alma Murlin, Mary Alyce Asbury, Geri Carter, and Gail Wathen that Drs Phil Edwards and Bill Ewing trained, starting just after the end of World War II. They had each become internationally recognized experts in the biochemical and serological identification of various genera of enterobacteria.

My research at CDC was no different than the work I had done at Walter Reed, however CDC had several substantial advantages. Their culture collection of unusual pathogenic bacteria was the best in the US, and probably in the world, each staff member was an expert in one or more specialty areas of enteric bacteriology, unusual cultures were sent to

Figure 4. At play when I could still jump, during the late 1970s in Atlanta.

CDC from all over the world, and dozens of the best laboratories throughout the world were anxious to collaborate with CDC.

When I arrived at CDC in 1974, there were no guards and essentially no locked doors, as evidenced by the substantial amount of equipment that "walked" out the doors (Figure 4). The laboratory and epidemiologic functions were in separate bureaus. There were 10 scientific positions in the Enteric Section and, excluding secretaries, there were no administrative positions at the section or branch level, and only one at the division level (that encompassed most of bacteriology). We were not allowed to apply for grants or to charge for cultures, and collaborations with industry were next to impossible to arrange. When I retired in 2000, there were scores of guards, every door was locked and everyone had identification badges and key card entry to a very restricted set of corridors. There were seven scientific positions in the section and eight administrative positions at the branch and division level. The number of administrative positions at levels above division had increased more than 20-fold. The amount of administrative work at the section level increased to a point that it occupied more than 50% of my time and significant amounts of time from every staff member. Much of the funding was from grants and cooperative agreements and we charged for cultures and some other services. Two other significant changes were that funding for CDC and its programs became more and more political, and CDC became the lead agency for bioterrorism.

I held a number of positions during this 25 year period, many of them caused by reorganizations (Figure 5). I was Chief of the Enteric Section (1974–1981), Enteric Reference Laboratory (1981–1982), Molecular Biology Laboratory (1982–1984), Meningitis and Special Pathogens Laboratory Section (1985–1993), Investigation and Surveillance

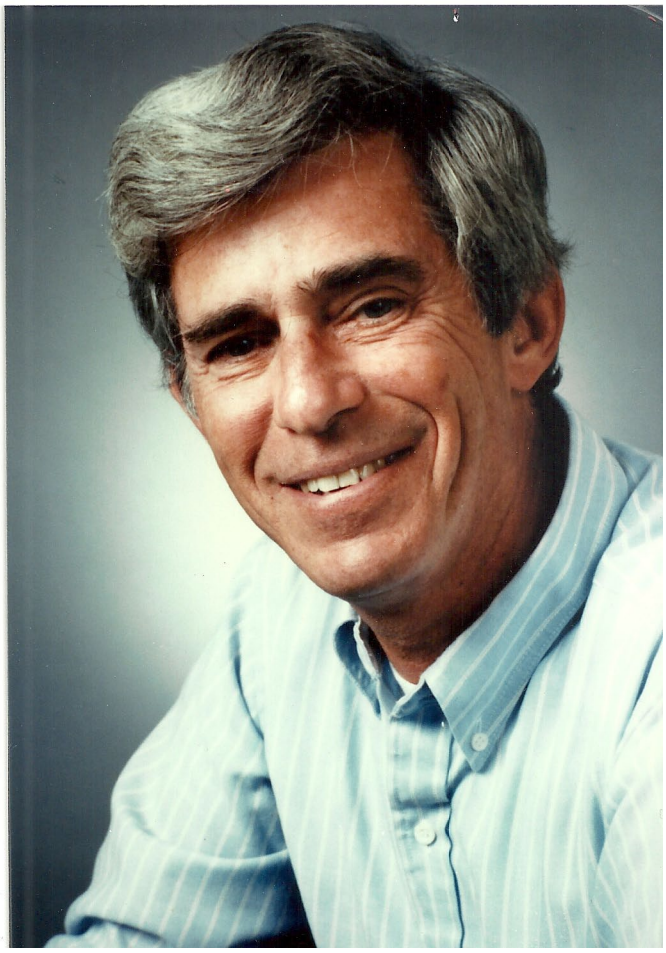

Figure 5. When the hair turned gray, just before glasses in the late 1970s.

Laboratory Section (1993–1997), Emerging Bacterial and Mycotic Diseases Branch (1996–1997), and Laboratory Section of the Meningitis and Special Pathogens Branch (1997–2000). My personal laboratory (which never had a name) was productive, averaging more than a publication a month and an abstract every 3 months. Since Arnie and I were its only employees, this could not have been done without a tremendous amount of help from dozens of collaborators, visiting scientists, and students (Figure 6). Perhaps the most credit should go to the unsung heroes of the public health system; the clinical microbiologists at the local and state levels who have the wisdom to recognize unusual isolates and the intellectual curiosity to follow up on them.

Although space and my failing memory prohibit citing all of our collaborators, many of them stand out. At CDC, in addition to Frances and the aforementioned laboratorians in the Enteric Section, there were Dr Jim Farmer (an authority on enteric bacteria and bacterial taxonomy), Dr Bob Weaver (an expert on bacterial identification of unusual bacteria), Dr Joe McDade (who discovered *Legionella pneumophila*), Dr Wayne Moss and Dr Maryam Daneshvar (both experts in

gas-liquid chromatography (GLC) who developed a genus specific identification pattern for legionellae and dozens of other genera), Dr William Cherry and Dr Roger McKinney (experts in *Legionella* and other serology), and Dr Claire Broome (an expert on just about everything and a wonderful supervisor). I have saved Dannie Hollis for special mention. Now in her late-80s, Dannie still works as a part time volunteer at CDC and one day a week at a clinical laboratory. Dannie's sweetness and humility are as large as the mountain of knowledge in her head. It is safe to say that she knows more about the biochemical identification of clinically significant bacteria than anyone in the world. She readily shares that knowledge and has been instrumental in the identification of several hundred emerging disease-producing bacteria!

It was a great privilege to collaborate with Stan Falkow. I learned a great deal from Stan, although I was never his student as many people thought. Stan sent us a number of students to train. Among them was Dr Jorge Crosa, a talented and delightful Argentinean. Dr Leon Le Minor, who was Dr Ewing's counterpart at the Institut Pasteur was among the first to recognize the potential of DNA hybridization for clinical bactertiology. He invited me to visit his laboratory and teach the method. One of the "students" was Dr Michel Popoff, who did much of the definitive work on *Aeromonas*. Drs Patrick and Francine Grimont, also of the Institut Pasteur were our friends and collaborators for more than 20 years. They trained with me and went on to outshine their teacher, both having spectacular, innovative careers working on enteric bacteria and in other areas. Another excellent scientist from the Institut Pasteur, who later immigrated to Israel, is Herve Bercovier, who collaborated with us on *Yersinia* and *Legionella*.

We trained Dr Jan Ursing who also worked on *Yersinia*, as well as *Campylobacter* and *Helicobacter*, and who introduced our methods to Sweden. Dr Barry Holmes, Curator of the National Collection of Type Cultures (NCTC) at the Public Health Service in England had been collaborating with Jim Farmer and extended his collaboration to me. He never used hybridization, but his biochemical studies and scientific acumen were instrumental in our studies on many groups of *Enterobacteriaceae* and *Vibrionaceae*. Drs Frits and Ida Orskov were Dr Ewing's counterparts at the Statens Seruminstitut in Copenhagen. Experts in *Escherichia*, *Shigella*, and *Klebsiella*, they contributed to our studies on these genera and greatly aided in the acceptance of our results by the medical community. Dr John Penner of the Canadian Center for Disease Control, shared his expertise on *Proteus* and *Providencia*, and played a pivotal role in the acceptance of our substantial reclassification of these genera. Dr Alex Von Graevenitz, head of the clinical microbiology department at the University of Zurich, asked if we would train one of his students, Dr Martin Altwegg. Martin and his wife Regula worked with us on *Aeromonas*, and introduced hybridization to Switzerland, doing further work on *Aeromonas* and other groups. Dr Jiri Schindler collaborated with us on *Citrobacter* and *Legionella* and he and his wife Emma became close friends and often dazzled us with their kindness and hospitality in Prague. He deserves special mention because, as a non-party member when Czechoslovakia was under Communist rule, he was denied travel and promotion, and still was the major driving force in keeping bacteriology up-to-date and finding support for younger colleagues. He was by far the person most responsible for staging three international microbiology meetings to bring foreign scientists and new developments to his country.

Figure 6. With Arnie and Frances in the lab at CDC in the mid-1990s.

I was able to hire six doctoral level employees during my 26 years at CDC. I knew two of them casually because they worked in other CDC branches, and knew nothing about the other four. With incredible luck, they all turned out to be superstars. Dr Fred Quinn is a molecular biologist who trained with Stan Falkow. With us he worked on cat scratch fever and other emerging diseases. After a reorganization at CDC he became a laboratory chief in another branch and then went to the University of Georgia to head the Clinical Microbiology Department in the School of Veterinary Medicine.

Dr Kaye Wachsmuth earned her PhD while working at CDC. We had an opening for someone with experience in molecular biology and hired Kaye. She worked on virulence factors in *Yersinia enterocolitica*, and used plasmid profiling to im-

plicate contaminated marijuana as the cause of a multistate outbreak of salmonella. As a result of another reorganization, Kaye went to another section where she quickly became its chief and then the Deputy Director of the Division of Bacteriology. From there she became a Deputy Director of the US Department of Agriculture.

Dr Tanya Popovic was working on enteric bacteria in another branch when we hired her. She worked on meningitis and, in addition to her research, immediately demonstrated a magnificent acumen for organization, national and international collaboration, and network building. She rose rapidly from laboratory chief to section and branch chief, to deputy director for science in one of the centers, to Deputy Director for Science of CDC within a 7 year span.

Dr Rob Weyant was hired to replace Dr Bob Weaver, when Bob retired as Chief, Special Bacteriology Reference Laboratory (SBRL), whose responsibility was reference identification of virtually all non-anaerobic bacteria other than *Enterobacteriaceae* and *Vibrionaceae*. In addition to carrying on and expanding the role of the SBRL, Rob was instrumental in studies to characterize species in many genera, including *Rochalimaea*, *Neisseria*, *Bordetella*, and *Leptospira*. He was responsible for accelerating and expanding the computerization of data from the tens of thousands of reference cultures housed in the SBRL. Together with Dannie Hollis, Wayne Moss and Maryam Daneshvar, he oversaw development of two manuals for the identification of unusual bacteria that contain not only biochemical data, but GLC profiles as well. Rob took the lead in the initial response to bioterrorism. He eventually left SBRL for the Office of Biosafety, and now is the Director of the Division of Select Agents and Toxins at CDC.

The odds were against our hiring Dr Bala "Swami" Swaminathan. He is a PhD bacteriologist who, along with some 25 MD clinical laboratory chiefs, applied for a clinical microbiologist position. We narrowed the field to five candidates, four of whom were clinical laboratory chiefs with outstanding credentials. Claire Broome was my supervisor at the time and we agreed to hire Swami – one of our best decisions. Swami played a pivotal role in the nationwide outbreak of listeriosis. He was also involved in diagnosing the agent of Brazilian purpuric fever, in subgrouping *Neisseria meningitidis* by rDNA restriction profiles, in characterizing the etiologic agent of cat scratch disease, and in characterizing and describing new *Bartonella* species. Following a reorganization (we had many of them), Swami was transferred and became Chief of the Enteric Bacteriology Section. It was there that he designed, perfected and implemented "Pulse Net". Pulse Net uses pulse-field electrophoresis to characterize bacterial isolates, computerizes the results and makes data known to state health laboratories. First used with *Escherichia coli*, the Pulse Net system allows rapid detection of interstate and international disease outbreaks. Now retired from CDC, Swami founded a company to provide consultation to local, national and international public health agencies.

Dr Leonard Mayer trained under Stan Falkow. Stan recommended him highly, which turned out to be an understatement. Leonard is a microbiologist and molecular biologist with tremendously broad and in-depth knowledge of a wide variety of diseases. His accomplishments include epidemiologic identification and characterization of pathogenic bacteria at the species and strain level, plasmid analysis to characterize foodborne disease outbreaks, and analysis of pathogenicity factors to elucidate mechanisms of infection and microbial resistance. The assay he developed for the rapid identification of *Bacillus anthracis* during the anthrax bioterrorist attacks saved thousands of people hours. He took similar decisive action as a key member of the Brazilian purpuric fever, Lyme disease, and listeriosis outbreak task forces. He and his colleagues developed dozens of non-radioactive assays for the rapid and sensitive detection of pathogenic bacteria, their toxins and other virulence factors. These have notably been used for the SARS outbreak, meningitis and encephalitis surveillance, and course development. One result of his extensive work in China is the revelation that *Haemophilus influenzae* b is a meaningful causative agent of meningitis, which led the Chinese Health Ministry to initiate vaccination in children (a total of some 20 million doses/year). Leonard and I worked together for 17 years and remain close friends. He remains at CDC, as a laboratory chief.

With these staff members and collaborators, it is not surprising that our laboratory was productive. Our 335 publications included some 280 research papers and about 40 review articles and chapters on new or unusual bacterial pathogens, bacterial classification and identification in journals and books such as

Bergey's Manuals of Systematic and Determinative Bacteriology, *The Prokaryotes*, and the *Journal of Clinical Microbiology*. In the course of our work we described more than 150 named species including some 60 new species of *Enterobacteriaceae* and more than 40 *Legionella* species, plus 20 new genera, scores of unnamed species and dozens of new subspecies. We also showed that a large number of atypical strains or serotypes either belonged to or should be excluded from a given species.

Many of our studies were initiated to either circumscribe the etiologic agent of a disease outbreak and/or to describe new or unusual disease agents. The best known of these is the emerging disease, legionellosis. We were able to show that this pneumonia could be caused by a large number of species (now over 50) belonging to the single genus, *Legionella*, and that there had been unnamed strains of the first species, *Legionella pneumophila*, first isolated 30 years previous to the strain that caused the infamous hotel outbreak in Philadelphia. Other examples are determining the agents of the Lyme disease and Brazilian purpuric fever outbreaks, and of cat scratch fever.

There are many examples in which we defined new virulent species "lumped" within species with less or no virulence. One such is *Enterobacter sakazakii*, a significant cause of neonatal meningitis, that had been included within *Enterobacter aerogenes*. Another is *Vibrio vulnificus*, a cause of fulminating septicemia after consumption of raw or undercooked shellfish or entry through wounds, that was mistakenly viewed as a biochemically atypical member of a nonpathogenic species.

Not all of our studies were on human pathogens. The reasons for this are two-fold: you must know the identity of the nonpathogens in order to define the pathogens, and non-human pathogens are frequently of substantial veterinary, agricultural or other economic importance. We demonstrated that epidemic septicemia in farmed catfish is a new species, *Edwardsiella ictaluri*, that two new *Vibrio* species caused so-called winter ulcer in salmon, and implicated several new *Erwinia* species in the destruction of many food crops.

Before the availability of so-called computer taxonomy and molecular identification methods, taxonomy was often a science in name only. There were no universally accepted methods for identification or for speciation. Organisms of interest to a given specialty were classified by members of that group. The methods used from group to group were often substantially different. For example, bacteria pathogenic for humans were the domain of clinical bacteriologists and they were speciated mainly on the basis of serology and pathogenicity. Plant-pathogenic bacteria were almost exclusively studied by plant pathologists and they were speciated largely on the basis of their host range, and so on.

We believed that since DNA contained the genetic material for all bacteria, it might be possible to arrive at a single, universal genetic species definition. Three things were necessary in order to test this theory. A rapid, relatively simple, reproducible assay to allow repeated testing of a large number of strains, type and well-characterized strains so we were reasonably confident that they represented a given species, and a working definition of a species based on DNA relatedness.

It had already been shown that DNA hybridization was a sensitive and reliable tool. It had been successfully carried out on filters and in agar, and now was being done on heated hydroxyapatite columns [at the proper salt concentration, double-stranded (hybridized) DNA bound to the hydroxyapatite, but single-stranded (unhybridized) DNA was eluted from the column]. When the temperature of the column was increased enough to denature the hybridized DNA, it was eluted from the column. This allowed us to determine not only the percentage of related DNA from two different strains of bacteria, but also to determine the thermal stability of the related DNA, which had a direct correlation to the percentage of unpaired bases within the hybridized DNA. However the columns were slow and it was difficult to run more than one or two at a time, which severely limited the number of tests. To overcome this difficulty, we made some simple modifications to the method, converting it to a batch procedure. We were then able to do 10 tests simultaneously and from 30 to 50 tests daily.

It was usually a simple task to obtain type and well-characterized strains from the collections at CDC, the American Type Culture Collection (ATCC), and from Barry Holmes at the NCTC in Colindale, England. The most important consideration was to see if, in fact, strains of a species could be defined on the basis of DNA relatedness to the exclusion of strains of all other species. We selected *E. coli* as the test species because it is probably the best studied of all bacteria. Well over 100 strains, were selected to maximize diversity. There were fresh isolates, isolates over 50 years old, strains with virulence factors, strains from diarrheal disease, urinary tract disease, and meningitis, strains from a variety of serotypes, and nonpathogenic strains. Regardless of origin or history, at an incubation temperature optimal for reassociation, DNA from all of the strains was well over 75% related to DNA from the type strain, and the thermal stability (indicative of sequence divergence where each degree of instability equated to 1% divergence) of the related DNA from all heterologous reactions was within 2 degrees of the thermal stability of the homologous reaction. Interestingly, the type and other strains of *Shigella* species showed equally high relatedness and thermal stability to *E. coli*. Type and reference strains of all other *Enterobacteriaceae* tested were significantly less than 70% related to *E. coli* and the thermal stability of the related sequences was substantially more than 5 degrees. Significantly, at a supraoptimal (stringent) incubation temperature at which only very highly related sequences can hybridize, there was little or no drop

in relatedness among the *E. coli* (and *Shigella*) strains, but a substantial decrease in relatedness to *E. coli* was seen with all other *Enterobacteriaceae*.

These results indicated that it was possible to identify strains of a species, at least in *E. coli*, by DNA relatedness, and, if we believed that conclusion, all *Shigella* species and *E. coli* are a single genetic species. We expanded the study to species in most of the genera of *Enterobacteriaceae* and to species in other families, with essentially similar results. It seemed clear that a species could be defined as a group of strains whose DNA relatedness was 70% or higher at a temperature optimal for reassociation and whose related sequences showed 5% or less divergence. A corollary of this definition was that relatedness at a stringent incubation temperature remained at or above 60%.

Sometimes two or more genetic subgroups were clearly distinguishable within a species defined by DNA relatedness (genomospecies), usually accompanied by biochemical differences. In these cases two or more subspecies were described. If a new genomospecies contained only one strain, especially when there was no biochemical data with which to differentiate it from other species, it usually would not be named (e.g., *Citrobacter* genomospecies 10, 11, and 12), which would be given formal names when additional data became available (e.g., *Citrobacter gillenii* formerly *Citrobacter* genomospecies 10).

Others have suggested that species should be defined on the basis of "polyphasic taxonomy," a combination of phenotypic and genomic characteristics, and more recently there has been an unfortunate tendency to classify bacteria solely on the basis of rRNA gene sequencing, frequently after examining only a single strain. While we agreed that it was preferable to use as much phenotypic and other data in describing a species, we know of no instance in which a species defined by DNA relatedness was changed or negated by phenotypic data. rRNA gene sequencing is an extremely powerful tool with which to define higher taxa, and, most of the time, species. However, others and we showed that rRNA gene sequencing is far from foolproof at the species level and that it is risky to use in the absence of other data.

We were confident of the species definition and committed to the tenet that, to be scientifically valid, a bacterial species should have the same meaning for all bacteriologists. Each specialty group of bacteriologists (clinical, plant, animal, industrial, environmental, genetic, etc.) has important special needs for communication, but these are not sufficient for, nor do they justify speciation. Pathogenicity, host range, virulence, specific mutations, serotype, and many other traits of paramount interest to some bacteriologists should not be a justification for creating a species. In other words, *E. coli* should mean the same thing to the clinical bacteriologist as to all other bacteriologists. To test this belief, we systematically (no pun intended) examined species from genera of *Enterobacteriaceae* and *Vibrionaceae*, and later, *Flavobacterium*, *Neisseria*, *Francisella*, *Bordetella* and others.

In *Enterobacteriaceae* the trend had been to divide (split) pathogenic species and to combine (lump) nonpathogenic species. *E. coli* and the four *Shigella* species are a single genomospecies. It is clear that they overlap in terms of virulence factors and pathogenicity, especially *Shigella* and the enteroinvasive strains of *E. coli*. *E. coli* strains show wide differences in disease-producing potential and virulence factors. Various strains cause invasive and toxigenic gastroenteritis, urinary tract disease, meningitis, and wound infections, and we all have *E. coli* as a normal intestinal inhabitant. All of these strains are definitely the same genetic species.

In *Salmonella* the three named species were shown to belong to a single species with seven subspecies. *Erwinia* species were split on the basis of pathogenicity and host range. Conversely, *Citrobacter freundii* contained strains that were shown to represent at least six other *Citrobacter* species. *Yersinia* contained both lumped and split species. *Yersinia pestis*, the causative agent of bubonic plague was split from *Yersinia pseudotuberculosis* (which causes plague-like illness in animals), whereas more than a half-dozen species were hiding under the umbrella of the less pathogenic *Yersinia enterocolitica*.

When these data were published, some changes were readily accepted and others were resisted by clinical bacteriologists and infectious disease specialists. Our naive notion that scientific data should dictate appropriate speciation and nomenclature was dispelled when we published the *Yersinia* results. *Yersinia pseudotuberculosis* was described prior to *Yersinia pestis*, which meant that it was the type species of the genus, and since the two species were clearly a single species, the species name had to be *Yersinia pseudotuberculosis*. Realizing the problems this could cause to the public health community we created two subspecies, *Y. pseudotuberculosis* subsp. *pseudotuberculosis* and *Y. pseudotuberculosis* subsp. *pestis*, and stated that subsp. *pestis* could be referred to simply as *Y. pestis*. This was deemed insufficient and in response to a "Request for an Opinion" to the Judicial Commission, our conclusion was overturned. The Judicial Commission ruled that, despite the validity of the science, *Y. pseudotuberculosis* subsp. *pestis* was a "dangerous name", due to the medical importance of plague and the fact that

quarantine regulations use the name *Y. pestis*, so they conserved *Y. pestis*.

The classification of *Vibrio cholerae* was, taxonomically, a figment of epidemiologic imagination. There are some 160 different O serogroups of *V. cholerae*, but only serogroup 01 had been implicated in large epidemics of cholera. Despite the fact that the majority of other O serogroups had been isolated from cholera cases, only serogroup 01 was called *V. cholerae*. All other serogroups were referred to as "noncholera vibrios" or "non-agglutinating vibrios". Both of these terms are totally misleading. All of the strains did agglutinate and many did cause cholera. Our results indicated that regardless of serotype, all of these strains were *V. cholerae*. In this case, Dr Paul Blake, a cholera expert and chief of the Enteric Bacteriology Branch, championed our results, and largely because of his influence they were readily accepted. Other new *Vibrio* species were defined, most from the marine environment. Some, such as *Vibrio* vulnificus, are quite pathogenic.

Bordetella pertussis, *B. parapertussis*, and *Bordetella suis* were shown to be a single species, split on the basis of their host range. *Francisella tularensis*, the causative agent of tularemia was shown to be the same species as *Francisella novicida*, which, although usually isolated from water, has been isolated from cases of tularemia. When we confirmed that the two pathogenic species of *Neisseria*, *N. meningitidis* and *N. gonorrhoeae* were identical at the species level, it surprised us and the medical community. They cause totally different diseases and provide additional strong evidence that pathogenicity differences do not necessarily correlate with species differences. *Leptospira* is another genus in which the most prevalent pathogen, *L. interrogans*, was distinguished from the "nonpathogenic" species *L. biflexa*, that we showed was a repository for many other *Leptospira* species.

I would have retired much earlier if I had a dollar for every time I heard someone decry a nomenclatural change saying that the old way worked fine, why did the taxonomists have to complicate it. If you think about it, all microbiologists either depend on taxonomy or do taxonomy. It is not only scientifically correct, but logical that a species means the same thing to everyone. While it certainly is true that each specialty has its own needs, they should not be expressed at the species level. It is possible to distinguish between strains of a species by subspeciation, or better to distinguish them by infrasubspecific designations, such as pathotype, serotype, phage type, etc.

In addition to describing new species responsible for emerging bacterial diseases, and addressing the validity of existing speciation, we had occasion to deal with nomenclatural problems. The Bacteriological Code requires that a type strain be designated for each species and that it be deposited in a recognized culture collection like the ATCC or NCTC. The type strain is usually the first isolate and the description of the species (rightly or wrongly, since not infrequently, the type strain is atypical) is based upon the type strain. During the course of our studies, Jim Farmer, Frances Hickman Brenner and I were quite surprised to learn that many well-described species did not have a type strain. We published a paper designating 19 neotype strains (a type strain designated after the fact) and added a few more in separate publications.

Just as there is a type strain for a species, there is a type species for a genus and a type genus for a family. The type genus of a family is the first genus described for that family (e.g., *Legionella* for *Legionella*ceae, *Vibrio* for *Vibrionaceae*, *Aeromonas* for *Aeromonadaceae*). The type genus of the family *Enterobacteriaceae* is *Escherichia*. Therefore, according to the rules of nomenclature, the family name should have been "*Escherichia*ceae". In addition, according to the rules, a family name is constructed by taking the genus name and adding the ending "*-aceae*". Therefore the correct name should have been "*Enterobacter*aceae". Aware of this, a request had been made to "conserve" the family name *Enterobacteriaceae* because it was so well-known. The Judicial Commission issued an opinion granting conservation of the name. When, several decades later, the Judicial Commission reversed itself, proposing that "*Escherichia*ceae" become the name of the family, we (Bill Ewing, Jim Farmer and I) objected to their proposal. Our objection, which eventually was approved, was based on the fact that these nomenclatural problems were known when the Judicial Commission first ruled to conserve *Enterobacteriaceae*. If the Judicial Commission were to reverse itself in the absence of very persuasive *new* data, it would negate its purpose and authority and open all of taxonomy to justified ridicule.

We were not involved in the final nomenclatural decisions in the genus *Salmonella*. These were made by Leon Le Minor, Michel Popoff and our other colleagues at the Institut Pasteur. However, our data were the basis for these decisions. Historically, *Salmonella* had been divided into four subgenera. Subgenus 1 contained some 1500 named serotypes that included almost all of the medically important strains (*S. typhi*, *S. typhimurium*, *S. enteritidis*, *S. paratyphoid*, etc.), subgenera 2 and 4 contained biochemically atypical strains, and subgenus 3 contained mainly animal isolates. There were three recognized species, the type species *Salmonella*

Figure 7. Receiving a CDC award from Dr Claire Broome.

typhimurium, *S. typhi* the cause of typhoid fever, and *S. enteritidis*, representing all other serotypes. Our studies indicated that each of the four subgenera were subspecies and identified two additional groups, one at a subspecies level and another that represented a second species. The accepted nomenclature is now *S. enterica* to represent all strains of the 5 subspecies and *S. bongori* to represent the few strains of the second species. Thus *S. typhi* is formally *S. enterica* serotype Typhi, *S. typhimurium* is formally *S. enterica* serotype Typhimurium, etc.

There were three studies that we did not complete, and still have not been completed. The first was on atypical *Vibrio* strains among which we identified more than a dozen new species. It lacked some confirmatory biochemical tests that would have helped phenotypic differentiation of some of the new species. *Flavobacterium meningosepticum* is a highly antibiotic resistant organism that has been reported to cause neonatal meningitis, as well as nursery outbreaks of this disease. In collaboration with Barry Holmes we compared some 50 strains that had been identified as *F. meningosepticum* to the type strain. Only one of these strains showed species-level relatedness to the type strain! The other strains represented some 30 different species! Due to the seriousness of the disease and the antibiotic resistance of the causative organism(s), it is important to determine whether the type strain is atypical, and which of the other genomospecies are pathogenic. We are still hopeful that Barry will complete this study.

The last uncompleted study was the most comprehensive and one of the most important projects during my tenure at CDC. It was a comparison of bacterial strain identification by routine biochemical tests and by rRNA gene sequencing. If neither method identified the culture or if different identifications were obtained, identification by DNA hybridization was used as the gold standard. There were two sets of test strains. The first set contained type and reference strains; the second contained a combination of other well-characterized strains in the CDC culture collection, and newly received, unidentified strains. In addition to directly comparing these methods, which had never been done, and evaluating the specificity of rRNA gene sequencing on multiple strains from a given species and from closely related species, the study was designed to determine whether rRNA gene sequencing was appropriate as a screening tool in large laboratories. Our initial results were quite promising, as both methods agreed more than 90% of the time. The study was coordinated initially by Rob Weyant and later by Maryam Daneshvar, both of whom were hamstrung by lack of personnel. Unfortunately it has been on the back burner for many years.

I very much enjoyed my years at CDC. Our service function as a reference laboratory for state health and federal laboratories was valuable as source material for our research projects and greatly appreciated by them. I loved the research challenges. I respected and admired the professionalism and expertise of my co-workers. I learned a great deal from them and from most of our collaborators around the world, many of whom became life-long friends. Finally, I worked well with my supervisors, both the PhD microbiologists and the MD infectious disease epidemiologists. They were always strong supporters of our work. I was totally committed to almost all of our projects and I always tried to provide strong justifications for them. The laboratory was always productive, which, as a supervisor myself, I knew was satisfying for my supervisors. They were all highly competent and well-versed scientists. Claire Broome, however, was exceptional. She was extremely knowledgeable and really made you justify projects, personnel, and resources, but once convinced, she was a strong advocate (Figure 7).

Not everything, however, was peaches and cream. There were two distasteful scientific incidents, both illustrating the pressure that can accompany scientific research and the ego requirements of some scientists. In one case we were collaborating with a researcher in another CDC division on a new species isolated in an outbreak of an emerging disease. It had been a somewhat contentious arrangement, and therefore we met and promised each other not to publish without the knowledge of the other. Shortly thereafter he sent out a publication for journal review. When I heard that he had submitted his paper, I rapidly submitted my paper. Somehow, both papers were cleared at CDC and were sent to the same journal. Fortunately, saving much embarrassment for CDC, the same reviewer got both papers and returned them with some very strong admonishments. Needless to say we never

again interacted with this person.

The other problem came during the massive efforts following the first outbreak of legionellosis. At that time, CDC had a Bureau of Laboratories and a Bureau of Epidemiology. The two bureaus did not closely interact and both were jealous of their turf. Cultures of suspected legionellae came to the Bureau of Laboratories from the state health laboratories and to the Bureau of Epidemiology from state epidemiologists and from CDC epidemiologists who were investigating cases. Our laboratory received all of the isolates because there was no laboratory in the Bureau of Epidemiology that was set up to do the DNA relatedness studies. It was a very messy situation involving the four or five species isolated after the type species, *L. pneumophila*. We were collaborating with a large number of laboratorians, while some of the epidemiologists were collaborating with a group at a university where some cases occurred. We were asked to defer to them and refused. Members of their group and one or more university officials visited and made several proposals that we rejected. Among them was to have us write the paper using their species names and naming a species after me (which I never wanted) if we deferred to their publication. The result was that we published and then they published using our species names but with two new generic designations. Their generic designations long ago fell into disuse. This was an embarrassment to CDC and an affront to my colleagues who actually suggested the names. This type of work at cross-purposes can no longer occur since the two bureaus were subsequently merged.

I guess that two acute problems in the course of a 26 year research career at CDC is less than one might expect, although each took its toll at the time. There were three other problems that were chronic, festering wounds that worsened with time. Before I delineate these, let me emphasize that almost every laboratorian that I know at CDC shares my sentiments.

There must be a disease that affects CDC Directors called "reorganizationitis". It seemed that every time we had a new CDC Director, Center Director, and sometimes Division Director there soon was a massive reorganization. These reorganizations were always done from the top down, with little or no input from the people who actually did the work. After each reorganization, responsibilities were changed at the lower organizational levels. That is the reason that I had so many different job titles. Then there was always the requirement to provide the new administration with virtually the same information required by the previous administration, but in a different format – a colossal waste of time. The second chronic problem was that each new director hired more administrators, who in turn, sought more information and required more administration from everyone below them, thus robbing section and laboratory chiefs of a significant amount of the time they should have been using for diagnostic service and research. I always believed that a prime responsibility of administrators was to facilitate, rather than hamper the work of the scientists – if you hire a scientist, let him/her do science. Finally, institutional memory was allowed to reach a dangerously low level. Not only were the number of supertechnicians substantially reduced, but when recognized world experts were nearing retirement, rarely were people brought in as apprentices to them. Worse yet, frequently these experts were not replaced at all (when I retired they did not replace me for 4 years!) and the work was divided among people who were already overworked and often undertrained. The lag time for receiving results from diagnostic reference identification services, which were once the crown jewels of the CDC bacteriology laboratories, grew so long as to often be useless to the submitting laboratory. Some laboratories have a backlog of up to a year! These problems are included not only because they affected my work and the work of everyone in my group and because they still exist, but because they are central to my regrets, which are addressed below.

In addition to formal duties, I had many informal responsibilities, some of which were drudgery and some were labors of love. I enjoyed presenting our work and had many chances to do so at national and international meetings, symposia and seminars. I also enjoyed teaching, both in the classroom and one on one with visiting scientists and graduate students. Reviewing manuscripts for science journals was a mixed blessing, as was editing. It was a pleasure to review and edit the excellently conceived and well-written manuscripts and painful to spend time on poorly executed studies and poorly written papers.

Another mixed blessing was the several years I spent as an Equal Employment Opportunity (EEO) counselor. I was the only counselor with a doctoral degree, so I was usually asked to handle cases in which the complainant and/or the accused held a high level position. It was time-consuming and sometimes frustrating, but also sometimes highly rewarding.

I was a member of many taxonomic and other bacteriology subcommittees that ranged from excellent to indifferent. The one that occupied most of my time and was by far the most rewarding was Bergey's Manual Trust. The Trust was created to publish *Bergey's Manual of Determinative Bacteriology*, which is now in its 9th edition. More recently, while I was on the Trust the 1st edition of *Bergey's Manual of Systematic Bacteriology* was published and the second

Figure 8. A Bergey's Manual Trust meeting, with, from left to right, Drs Peter Sneath, Noel Krieg, Jack Holt, Bob Murray, Jim Staley, Norbert Pfennig, Don Brenner and Marvin Bryant.

edition was partially completed. Also, Bergey's Trust recognizes individuals who made outstanding taxonomic achievements through the Bergey Medal and the Bergey Award. The Bergey Trust stimulates advancement of taxonomy not only by publishing the *Manual* and other taxonomic publications, but by sponsoring symposia, and stimulating taxonomic research in other respects. The Trust has a board with nine trustees. The trustees usually meet once each year, with much of the work accomplished at the trustees home institutions and by the editor of the Trust and his staff at the editorial office. The Trustees are elected and serve until they retire or reach the age of 70. I was elected in 1979 and served as secretary from 1985 through 2000 (Figure 8). I authored or coauthored a number of chapters for the manuals, edited scores of chapters submitted by invited authors, and was a co-editor of a volume of the latest *Manual*. The Trustees are all internationally recognized authorities. I regarded myself as a neophyte on this august panel; a token medical/molecular type if you will.

Even among this panel of experts, there were three who stood out as mentors for me. Dr Jack Holt, who for 20 years or more served both as editor and treasurer of the Trust, accomplished an incredible amount of work without fanfare; Dr Peter Sneath, the world's leader in numerical taxonomy, who brought total objectivity to any problem and who was a "doctor of philosophy" in the original sense, and Dr Bob Murray, who, as Chairman of the Trust for many years, always managed to get a consensus from this strong-willed group, and remains another consummate "doctor of philosophy," extremely knowledgeable on virtually everything in and beyond science. They each had a profound influence on me both scientifically and personally. Worthy of special mention is Joan Sneath (now deceased), whose kindness to me and to Frances made us feel welcome and wanted.

I had never taken a retrospective look at my career. Now forced to do so, as a taxonomist, how would I and how did others assess the successes and failures in the body of work? One measure of acceptance by your peers is election to honorary societies. I was fortunate in this regard, being elected to membership in a number of prestigious societies and being elected to honorary membership in the French Academy of Microbiology and the Microbiological Society of Bohemia and Slovakia. Another measure of peer acceptance is in an award received for your work. I received or shared in a number of national and international awards and honors, as well as awards from CDC and the Department of Health Education and Welfare. While it was a great honor to receive or share in awards, I am well aware that any award I received was the result of the efforts of many, and that those who receive awards are not always the most deserving.

I do believe that we made a contribution to taxonomy by developing and strongly advocating a single approach to bacterial speciation, even when our conclusions did not prevail. Our work brought a greater awareness of taxonomy and its importance to a wider group of bacteriologists. We trained many young taxonomists and hopefully had some influence on others choosing to work in the field. I am proud of the scientists we trained, many of whom became international authorities. Our work on disease outbreaks, including legionellosis and other diseases caused by emerging pathogens is also worth noting. As I look back, however, it is my failures that are most vivid.

DNA hybridization has repeatedly been criticized as a method that is too complex and cumbersome, and therefore only a very few laboratories use it. This criticism has often been used as justification to abandon it as the gold standard for bacterial speciation. I have disputed this, since I was never convinced that sequencing or any other method was as accurate. However, we never attempted to automate the procedure. I know that we could have created an array system using a non-radioactive probe that would have made it simple to complete hundreds of assays daily. As I said, our

Figure 9. Mixed doubles can be fun.

Figure 10. Retirement is wonderful.

laboratory was a two-man operation and we had no time to devote to automation. However, I never tried other options to obtain the additional personnel.

I regret that I was not a better supervisor. One of the weaknesses in the government system is that people are rewarded for good performance and often promoted until they reach a level of incompetence. Supervision, especially for bench scientists, like parenthood, usually comes without an instruction booklet. Many bench scientists are terrible administrators and supervisors. I was a pretty good administrator (despite despising administrative duties), but only a mediocre supervisor.

Another of my regrets was a lack of diplomacy. My supervisors and colleagues knew that I would give my honest opinion without any sugarcoating. The same was true in my presentations and research papers. I know that this tendency was sometimes counterproductive in my efforts to gain acceptance of our taxonomic points of view. If I had been more persuasive, we might have obtained more in-house support for our programs and more international acceptance of them.

I regret not being more forceful and vocal in opposing the ever increasing administrative load placed on all of the branches, sections and laboratories. I should have made it my business to express my (and everyone else's) dissatisfaction with the amount of "busy work" demanded by the upper level administrators, and in the all-to-frequent tendency for administrative policies and personnel to make things more difficult, rather than easier for the laboratory scientists, and I should have made stronger arguments for adequate personnel in the diagnostic reference laboratories.

Finally, I regret that the three studies in progress when I retired were never completed. The nearly completed *Vibrio* and *Flavobacterium* studies provided data to clarify the status of these two diverse genera, which, I believe would be of value to clinical and no clinical laboratories. The study of identification of bacteria by traditional biochemical tests, by rRNA sequencing, and by DNA hybridization is a comparison that is sorely needed to make long-term diagnostic and taxonomic decisions.

Many scientists really never retire, mainly because work is the single most important driving force in their lives. Others "retire," but remain as busy or busier than ever with other jobs, consulting, editing, etc. When I was asked what kind of work I would do when I retired, I replied that if I wanted to keep working I would not retire. I was ready to retire – work was beginning to interfere with the rest of my life. The moment I retired stress left my body like air from a punctured balloon. Both Frances and I play lots of tennis and go to as many tennis tournaments as we can (Figure 9). I take photos of tennis players and collect tennis memorabilia. My two post-retirement publications deal with tennis postcards (Figure 10).

Dedication

Dedicated to Wallis DeWitt: always selfless, always looking for a way to help, and always upbeat, even in the face of a horrible disease, and who personified the best traditions of CDC.

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Bergey's and me

My life with the prokaryotes

John G. Holt

In answering the request of the editor to relate some of my experiences in the arcane world of bacterial systematics, I have had to dredge up memories of a past life, long forgotten in happy retirement. Most of my professional life was spent in the service of *Bergey's Manual*, on and off, from 1960 to 2000. I occasionally performed "stand-up comedy" for my colleagues, relating stories of my shared life with so many wonderful people in systematics, and perhaps I will include some here. I will try to give the reader a flavor of the times in constructing bodies of work like *Bergey's Manual*. R. G. E. Murray and I covered the history of the *Manual* exhaustively in a chapter of that title in the introductory essays in Volume 2, Part A of the 2nd edition of *Bergey's Manual of Systematic Bacteriology* (BMSB). Please refer to that chapter for details that I will not include in this piece. Of course, I had a life apart from *Bergey's Manual* and I will share some of that with you.

Early life

I was born in 1929, 2 months after the crash (no relationship to the event) in Buffalo, NY, to working-class parents, and was an only child. My mother died in 1933 and my father remarried a few years later to a highly educated nurse (B.S. degree from the University of Chicago). She had had aspirations to become a physician, but her strict, sexist German-born parents, who told her to become a secretary, thwarted these. She compromised and became a nurse. She was a great stepmother and was very supportive of my interest in science. When I was about 12 she bought me a wonderful old microscope (with oil-immersion lens) from a retiring physician. It introduced me to the microscopic world. Unfortunately, I attended high school in Buffalo and public schools in that city did not offer biology, probably because of religious reasons. The school superintendent later retired to become a priest in the Roman Catholic Church. Biology would have to wait until college.

College

After graduating from high school in 1947 with a New York State diploma in college prep, I applied to Cornell University and was accepted to enter in 1948. I had to wait while the university preferentially enrolled returning veterans, so I worked for a year in a factory making parts for electric motors, before entering the school. At Cornell I majored in Food Technology in the College of Agriculture. I had been advised to be in that college which is state-supported and therefore, at that time, was tuition-free for New York State residents. I had wanted to major in chemistry so the college put me into Food Technology presumably as the "closest thing".

One of the required courses for Food Technology was Introductory Bacteriology, a six-semester credit course with lab. The course was taught by James Sherman, who was Head of Dairy Industry, the home department of both bacteriology and food technology. One of my lab assistants was Harry Seeley, who authored the genus *Streptococcus* in the 8th edition of the *Manual*. I loved the course, especially the lab that was heavy on technique and identification, and relied on the 6th edition of *Bergey's Manual*. It was my sophomore year and I decided to take all the bacteriology courses I could. These included Sherman's Advanced Bacteriology (the lab mostly studied all the species of *Streptococcus*, Sherman's specialty), Georges Knaysi's Cytology and Mycology courses, and Eugene Delwiche's Physiology. This experience convinced me to become a microbiologist and my most ardent interest was in classification and identification of bacteria. These areas seemed to appeal to my aesthetic senses and it helped to have a good memory. Of course, I never dreamed I would end up editing *Bergey's Manual*.

Graduate school beckoned and I decided to make the switch from food technology to microbiology. After graduating from Cornell in 1952, I enrolled at Syracuse University majoring in microbiology. It was a good opportunity to add more courses in chemistry, genetics, botany and microbiology. I graduated in 1954 with an MS degree. From there I looked for programs that offered course work in taxonomy and found that the Department of Biological Sciences at Purdue University had a graduate course in bacterial taxonomy taught by Dorothy Powelson. I applied and was accepted in the spring semester, 1954. Powelson took me on as a graduate student and we decided I would work on a project studying the myxobacteria. I was her first student to work on this fascinating group of complex bacteria, and to

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start we needed pure cultures. I managed to isolate about 40 cultures of various species of *Myxococcus*, and we decided to concentrate efforts on a strain of *M. xanthus* for a study of its slime layer.

In 1955, I married Bess-Gene Gillespie, whom I had met soon after arriving in West Lafayette. She was working toward a PhD in child development and developmental psychology and we joined the ranks of married grad students. In 1958 our daughter Susan was born and we began a different style of life managing to balance childcare, studying, research, teaching and all the other tasks of young married couples. We both graduated in 1960.

Also in 1958, Dr Powelson took a sabbatical leave for a year and I was asked to teach both of her graduate courses: Taxonomy and Cytology. This was a wonderful experience and very good training for an academic career. I added to my teaching experience when I was hired part-time to teach microbiology to nursing students at a local hospital in Lafayette in 1959.

Early professional life

I started my professional career by taking a post-doctoral position at Iowa State University in Ames under the mentorship of R.E. Buchanan. He had recently taken over as editor and chairman of the Bergey's Manual Trust following the death of Robert Breed in 1957. Buchanan had himself appointed Research Professor and had a grant from the National Library of Medicine to continue the work of the Trust. Buchanan was in his late 70s and had made a number of important contributions to bacteriology. For example, he named the parts of the growth curve and put together an early classification of the bacteria in 1916 that was used by Bergey in the first and subsequent editions of his manual. He also wrote one of the best textbooks in use in the 1940 and 1950s. In addition, he was a remarkable administrator, concurrently holding the posts at ISU of Head of Bacteriology and Dean of the Graduate School from 1919 to 1948, and Director of the Agricultural Experiment Station from 1936 to 1948. The latter two positions made him the *de facto* dispenser of research funds for the university, and, combined with his foresight, led him to fund some important discoveries. Probably the most important was giving J.V. Atanasoff $650.00 to develop the first digital computer in 1939. Unfortunately, Iowa State did not patent the invention and missed out on untold amounts of royalty money. Mauchly and Eckert eventually used the idea in the 1940s, leading to the first mainframe computer, the ENIAC, based on Atanasoff's binary system.

Buchanan's current project was to continue the work of Breed of compiling an annotated list of all the names given to the bacteria since Linnaeus, with their synonymy, validity and legitimacy according to the Rules of Bacteriological Nomenclature (written mostly by Buchanan). This list was finally published in 1967 as *Index Bergeyana* and contained over 29,000 entries plus bibliography. A number of us worked on the book, checking on all the original publications of the names, 90% of which were in the wonderful collections of the Iowa State University Library. The work was tedious but interesting and certainly made us all good "Code lawyers". The book was useful until the publication of the *Approved Lists of Bacterial Names* in 1980, after which it functioned as a historical record. I was honored to be a co-author with Buchanan and Erwin Lessel.

Besides working on the Index I was also doing research on the myxobacteria and other gliders and teaching an introductory course, which satisfied my love of teaching. I also became involved in some early efforts in numerical taxonomy. Some of my colleagues in the department, Bill Lockhart and Paul Hartman, had a small project with an entomologist and were using Peter Sneath's newly invented numerical taxonomy. They were using the "Cyclone" computer, a student-built mainframe based on Illinois' ILIAC, to study a small collection of bacteria isolated from insects. This computer, powered by vacuum tubes and housed in an enormous room, had about 72K of RAM! Lockhart and I went on to collaborate on numerical studies of *Salmonella* serotypes and later amino acid producers. The latter study involved strains of the genus *Arthrobacter* that led me to further work on the genus for many years to come.

Working for Buchanan was, in many ways, a rewarding experience. He was a masterful writer of papers, letters and grants and had a great fund of knowledge and experience. He exuded administrative competence and, even though in his late 70s, one could imagine him being a strong and fair administrator. He was a native-born Iowan and had spent his entire life in the state and in the service of Iowa State. He was leery of my working in the state; because I was an easterner he wasn't sure I would stay. I allayed his fears by telling him (true) that my wife was born in Cedar Rapids, Iowa and at the same hospital as he. That settled the matter, although truth be told, my wife was much more unhappy with the university and Ames than I ever was. Buchanan, always thinking internationally, was singularly responsible for the superb library holdings, especially foreign, in microbiology. It was rare that we needed to borrow books or journals from other libraries; they were in the ISU library or in the combined Breed/Buchanan reprint collections. He had prescient foresight and welcomed new ideas and techniques.

Personally, he was difficult, formal (he only referred to and

Figure 1. Attendees of Bergey's Manual Trust meeting in Ames, Iowa, 1960. Front row, L to R: R. E. Buchanan (BMT and ISU), C. F. Niven, Jr (BMT), N. R. Smith (BMT), E. G. D. Murray (BMT). Backrow, L to R: L. Y. Quinn (ISU), R. J. Beers (ISU), J. G. Holt (ISU), E. F. Lessel, Jr (ISU), W. R. Lockhart (ISU), unidentified outside taxonomist, W. Clark (ATCC), P. A. Hartman (ISU), R. Hugh (GWU), T. Pridham (NRRL).

addressed males by their last names), and very possessive of his editorship. Interestingly, assertive females could easily subdue him. He had a way of asserting authority, even over some of the biggest names in microbiology, notably the powerful men who served on the Bergey's Manual Trust Board of Trustees. He did everything he could to maintain his editorship, even as he grew more and more infirm.

Buchanan was a teetotaler and was always disturbed by any drinking that went on at mealtimes during Bergey meetings. E.G.D. Murray especially would rib him about his abstinence. For instance, he once brought scotch-flavored candies (the actual brand was Buchanan's) to the meetings and would pass them out and kid Buchanan about having one. Once Murray told of a dinner at the 1936 London Congress during which the king was toasted with very old port from the Cambridge University cellars and Buchanan had to drink it along with everyone else. Murray ended the story with "What a waste of good port". Buchanan did not smile.

I was involved in the early meetings of the Trust and in 1962 or 1963 was appointed Secretary-Treasurer, a non-voting position. It was a very exciting time for me to meet such important and accomplished men. Wonderful people like E.G.D. Murray, Sam Cowan, N.R. Smith, and later Norm Gibbons, R.G.E. Murray, Roger Stanier, John Liston and Arnold Ravin, all members of the Trust during the early 1960s (Figure 1). The group sometimes met in Ames (not a preferred location as the town was dry) but more often a few days before the annual ASM meeting. Buchanan would compose a long, detailed list of agenda (he rightly pointed out that agenda is a plural noun) that was to constitute the items to be discussed. These discussions were interminable and never seemed to produce any meaningful action. My job was to produce equally detailed minutes of the ramblings, which I tried to do to the best of my limited abilities. Mercifully, we bought a "portable" tape recorder (reel to reel weighing about 10 lb.) and I was able to refer to the tapes to glean the material for the minutes. I always held on to the tapes thinking they might be of historic interest. I used them only once, many years later, to extract E.G.D. Murray's posthumous, wonderful, often acerbic, comments and compile them for his grandchildren and their families. Probably the original tapes have not survived the two subsequent moves of the Trust office.

Most of the discussion at these meetings involved the preparation for the 8th edition of the *Manual*. Topics such as who should author the various sections and how the *Manual* should be arranged. There were always disagreements between Buchanan and the others about big issues, e.g., higher taxa. Stanier, being a very assertive person, was especially troubled by Buchanan's intransigence and at one point told him "Buchanan you're interested in names, I'm interested in biology". Needless to say he got nowhere.

In 1966 I was at a turning point in my career. I had been appointed Assistant Professor in the department in 1963 and began developing my own interests while keeping a hand in the Bergey projects. My wife and I had two more daughters in 1961 and 1964 and had become active in University and community affairs. My wife had also been appointed to the faculty in the Department of Child Development. I had an active teaching schedule and continued some research efforts along with the usual committee work. Buchanan was becoming more difficult and it became obvious that he was going to hang on to the editorship and chair as long as he could and there was no hope that I would ever be asked to take over. So my best recourse was to resign as Secretary-Treasurer and divorce myself from Bergey affairs. This I did, much to Buchanan's chagrin, and other Board members took over my duties. By this time we had sent the manuscript of *Index Bergeyana* (hundreds of boxes of 5 × 8 cards) off to the publishers, Williams & Wilkins. I went on about

my business of being a faculty member, achieving tenure and promotion the next year.

I kept up my interest in numerical taxonomy, applying the technique to analyzing the diversity of bacteria in soil habitats, and also continued studying the gliding bacteria. One of Buchanan's graduate students, E.E. Jeffers, had enriched an interesting filamentous glider that he encouraged me to look at. I obtained three strains in pure culture and it turned out to be quite unique and, through correspondence with other colleagues, found that Ralph Lewin at Scripps Oceanographic Institute had isolated similar organisms. We jointly published a description of a new genus *Herpetosiphon*. Little did we know that later it would be classified in a new phylum. Lewin's strains later were shown to belong to a different genus and phylum

Our department (still called Bacteriology) was small with seven professors, and with strong undergrad and graduate programs. We were a tight-knit group and went many years before being able to add additional faculty. I loved teaching, especially with the very bright Iowans, and taught a number of different courses. Like everyone else I ended up on committees at all levels and, in particular, on curriculum committees, both college and university. These committees were very important at Iowa State, where the faculty had complete control over curricula and the courses that departments were allowed to offer. Also, in the 1960s and 1970s there was a great deal of experimentation and changes in requirements, which put me in the middle of the fracas. Interesting times!

My service also extended to the ASM, ICSB and the ATCC in various roles as editor, board member, secretary and committee member. Receiving the J. Roger Porter Award from the ASM and American Federation of Culture Collections in 1985 rewarded this service.

In the late 1960s the Board of Trustees had finally made progress on the 8th edition. They had wisely decided on a non-hierarchical format, dividing the book into Parts instead of Orders, etc. They had engaged a large number of authors from around the world to write chapters. They asked fellow Board member Norm Gibbons, who had recently retired, to do the major editing of the manuscripts. His co-editor R.E. Buchanan was responsible for the bibliography and ancillary material, all of which could be done in Ames by the secretary and clerks still working in the Trust office. In 1972 Buchanan was told, by either the Trustees or by Gibbons, to ask me to help proofread the galleys, which were beginning to pour in. He did, very graciously, and I likewise accepted. By this time he was in his late 80s, his wife was in a nursing home, and he was about to go there himself as a "guest" as he explained it. He died in January 1973 before the *Manual* was published. He had collapsed in bed in the nursing home (the bed was flanked by two bedside tables piled high with paperwork) and the personnel at the home related that as he was being wheeled out on a gurney to the ambulance, he momentarily revived, sat up and asked, "Who authorized my transfer?" Forever the administrator right to the end.

Back to the *Manual*

After the death of R.E. Buchanan I became *de facto* caretaker of the Bergey office. The office was abandoned, the staff retired and the large task of proofreading the galleys of the 8th edition was tackled and eventually finished in time for publication in the summer of 1974. About this time I was promoted to Full Professor, a welcome recognition of my efforts on behalf of the university and the field of microbiology.

I was voted a member of the Board of Trustees in 1973, which I gratefully accepted. I had been so involved in Trust matters for so long that I felt part of the Bergey "family" and I loved the work and the people involved. The Board of Trustees then consisted of John Liston (Chair), R.G.E. Murray (Vice-Chair), Arnold Ravin (Treasurer), Sam Cowan, Norm Gibbons, and Roger Stanier. It was at this meeting in Ottawa that Sam Cowan moved that the Trust set a mandatory retirement age of 70, no exceptions. It passed unanimously and was thereafter known as the "Buchanan amendment". It may have been a bit severe because the Trust lost the services of many people still in their intellectual prime. I do not include myself in this category; I was ready to retire from the Board at 70.

In 1970 royalties from the sale of the 8th edition came rolling in. It was the most money the Trust had ever seen and would go a long way towards supporting the Trust's programs for some time. Particularly noteworthy was the fact that about one-half of the sales were outside the US, making the book a truly international effort. With this in mind the Chairman, John Liston, suggested that the Board hold its next meeting outside the US (his original idea was a beachfront resort in the Caribbean, which, after discussion, was rejected) and after that alternate with the US. The Board had recently invited Danish microbiologist Hans Lautrop to be a member and he offered to host the next meeting at the Statenserum Institut in Copenhagen.

There were a number of changes in the makeup of the Board of Trustees during the 1970s. Quite a few members retired: Cowan in 1974, Stanier in 1975, Gibbons and Liston in1976, Lapage in 1978, and Lautrop in 1979. New members were

added: Marvin Bryant and Stephen Lapage in 1975, Noel Krieg and James Staley in 1976, Norbert Pfennig and Peter Sneath in 1978, and Don Brenner in 1979. These additions brought the membership to the maximum of nine and with it a continuation of the international composition to compliment the authorship and sales of the *Manual*. For me it was a pleasure to work with this distinguished, accomplished, and compatible group of colleagues.

In 1975 I was asked to be Editor-in-Chief, a singular honor and a task I was sure I could do well. I had a number of thoughts about the future of the *Manual*, for instance, the need for an inexpensive abridged edition and the inclusion of the cyanobacteria in the next edition. I remembered how useful the abridged 6th edition was when I was an undergrad student. It had been compiled by Breed and published by H.J. Conn, a Trust member, in 1948. It was paperback, inexpensive and very useful, containing keys to the genera and species. I proposed to the Trust that we abridge the newly published 8th edition, put it in a soft cover and sell at a low price (having student needs in mind). The idea was accepted and it was decided to call it the "*Shorter Bergey's Manual of Determinative Bacteriology*". It was a relatively easy task. I asked Williams & Wilkins, who had agreed to publish the book, to provide two copies of unbound pages of the 8th edition. I cut and pasted those sections I wished to include, e.g., the keys, tables and descriptions of all the taxa from genus to order. There were sections in the *Manual* that did not have good keys so I had to construct new ones. In some cases I had to put together synopses (as opposed to dichotomous keys) to help the user with a diagnosis. The book was published in 1977 and was successful, selling over 25,000 copies during its lifetime. It was subsequently translated into Russian and published in the Soviet Union in 1980. Williams & Wilkins had negotiated a contract in which the Russians paid royalties, apparently a first, though a meager remittance. We were indebted to George Zavarzin of the Institute of Microbiology in Moscow for seeing the project through to completion and checking the translation by S. Ter-Kazaryan.

At the same time we needed to begin planning on a 9th edition. We wanted to avoid the long period (17 years) that had accrued between the 7th and 8th editions. We also needed to respond to the explosive growth in new taxa that we knew would come about with the publication of the revised Code of Nomenclature. Of course, our concerns were justified in the 1980s and beyond, as there has been a very large increase in the description of new taxa. Also, the publications of Carl Woese and co-workers in 1978 stimulated this activity and indicated that we would finally begin to be able to construct a natural classification of the prokaryotes. Obviously we needed to include much more information and analysis of relationships than before. We asked Hans Lautrop to compile a list, extracted from the 8th edition, of the different kinds of information that should be included in each chapter. He presented his list and, after much discussion, we adopted his ideas and decided that after the description there should be sections on: further descriptive information, enrichment and isolation procedures, maintenance procedures, methods for testing special characteristics, and taxonomic comments. It became apparent that the book was going to be much bigger than the last edition, covering more information and that the determinative aspects were becoming secondary to a larger discussion of the systematics of each taxon. The title of the new edition had to be changed to reflect the new approach, and it was decided to call it *Bergey's Manual of Systematic Bacteriology*, Edition 1.

More problems surfaced: that all the information would not fit into a single volume and that the expanded book would be more expensive. My goal as Editor, supported by the Board, was to keep the price down as far as we could, keeping in mind that the book should be available in the developing world as well as North America and Europe. Our solution to these two issues was to split the book into volumes that could be purchased separately and that would appeal to large blocks of specialists. There would be four volumes: Gram-negatives of medical importance, Gram-positives of medical importance, other Gram-negatives (including the archaea and cyanobacteria), and the actinomycetes. Each of the volumes would have one or more editors and the final editing, bibliography and index would be handled in the Ames office. Publication of the volumes was to be on a cascading schedule over the next 4 or 5 years. A number of advisory committees were formed to advise the Board on the composition of taxa of each volume and suggestions of authors. This scheme had worked very well for the 8th edition and was expanded for the systematic volumes.

In 1980 our Treasurer, Arnold Ravin, became very ill and soon after passed away at a relatively young age. I was asked to assume the Treasurer position in addition to being the Editor-in-Chief. It was a desirable arrangement because I could write checks as needed, for supplies, travel, etc., and also deal with the annual Internal Revenue Service filings. To augment expenses that were not covered by royalty income, I applied for and received a grant from the National Library of Medicine for the first two volumes.

I was blessed with the help of the fine editors of each volume. Volume 1 was handled solo by Noel Krieg and was the first to start production and served as the trial run for the other volumes. Our numerous advisory committees had

suggested many potential authors and Noel invited them to submit manuscripts, giving them a generous deadline. To facilitate their efforts we put together an extensive set of instructions to authors with rigid rules and sample chapters, to ensure uniformity of presentation. Most authors followed these instructions, but when they deviated from the rules their submissions were corrected, and in some cases, redone by Noel. He and I stayed in touch by phone, solving problems, some minor, some major, that kept coming up. We had a few problems with authors. Some died (both literally and figuratively) during the process and had to be replaced. One author of an important genus was very late in submission and when Noel contacted him, the Chairman of his department answered and told him that the person had been institutionalized for severe mental problems. We were afraid that the Bergey task had sent him over the brink. Fortunately, Noel was able to conscript a new faculty member at his university, Virginia Tech, who was an expert on the genus, and who agreed to write the chapter on short notice.

All of this editing was taking place in the early 1980s, before email or good word-processing on personal computers. The immediate task in our office was to construct the voluminous bibliography. My part-time secretary, Cynthia Pease, was very competent and was willing to take on the task of imputing the bibliography into a computer. A programmer in the ISU Computer Lab helped us by writing a program that would sort the references alphabetically by authors, including multiples, and by date. No mean task! Mrs Pease typed in each entry on a monitor connected to the university mainframe through a 300-baud (300 bits per sec) modem. Williams & Wilkins was able to read the final output on magnetic tape and convert it to typescript. This sorting procedure was quite a technical feat considering there were thousands of references, some with as many as ten authors.

The finished manuscript was finally sent to the publisher in 1983 and the book was published in 1984. My goal was to produce a book that was as free of errors as possible, so I made sure that I had proofread the manuscripts and galleys very thoroughly. These were not the kind of books that could tolerate errors. There were, of course, opinions stated by the authors that were subject to debate, but these were the products of each author's personal ideas and we gave the authors the freedom to state their opinions. These usually occurred in the taxonomic comments and we felt they would eventually be sorted out and would possibly stimulate more discussion. That is what makes taxonomy such an interesting field and why I enjoy it so much.

The other three volumes were on schedule following our plan of cascading production. Volume 2, edited by Peter Sneath came out in 1986; Volume 3, edited by Jim Staley, in 1989; and Volume 4, edited by Stan Williams, in 1989. There were 290 authors from 19 different countries who gave a much-appreciated effort and whose only compensation was a free copy of the volume in which their chapter appeared. The editors and their associate editors (and myself) all had other onerous duties, usually professorships at research universities. The Trust was indebted to them for their time and for their employers for providing space and time. I might add that I proofread the whole book, including references and indices, making me the only person in the world to read the book cover to cover. No one else would be crazy enough to try. The book was well received and each volume sold extremely well.

Let me say a few words about managing the Trust affairs. The early Editors-in-Chief, who were also chairmen of the Board of Trustees, took an authoritarian approach to running the operation. We felt that that style was not good and so separated the functions. We envisioned the operation as one closer to the US public school plan (or also corporations, but on a smaller scale), where there was a School Board with its president and other officers (= the Board of Trustees), and the Superintendent of Schools (= the Editor-in-Chief). The former decides policy and the latter runs the operation and presents the Board with plans and activities. (I use this example because my wife and I spent some time hassling the Ames School Board and had to understand the power structure). It was my job with Bergey's Trust to organize and compose the agenda for the meetings, manage the resources, and be the liaison with the publisher. In general, keep the operation on an even keel. Our overall goal was to produce useful, affordable, up-to-date encyclopedic treatments of the prokaryotes that were free of errors and undue controversy (but with enough to stimulate more research).

Post-1987

The late 1980s was a watershed time for the Bergey operation and me. First of all, my wife and I needed a change. Our three daughters had "left the nest" and were basically on their own and living away from us. We were getting sick of the extreme weather in Iowa and my wife, who suffered from severe rheumatoid arthritis, needed a change. She had been writing books and consulting with the federal Head Start program, but that work was drying up.

The situation at Iowa State was changing for the worse as far as microbiology was concerned. The University had a new president after 19 years, and he wanted to make changes. He appointed a committee to review and make suggestions about the organization of colleges and departments. Their

subsequent report did not bode well for our small department. We were a small unit in the College of Sciences and Humanities. We had been unable to grow in number of faculty and the report recommended the department be broken up (along with the Botany, Zoology and Genetics departments). I would have ended up in a department consisting mostly of fish and wildlife biologists, plant ecologists and taxonomists who still considered the bacteria to be part of the Plant Kingdom. I saw the handwriting on the wall, so to speak, and knew it was time to leave.

About this time in June 1987, Jim Staley and I were invited to visit the USSR as guests of the Academy of Sciences and the Institute of Microbiology, where we had a number of important contributors. George Zavarzin facilitated our trip and we spent a delightful week there. This was my second trip to Russia since I attended the Moscow Congress in 1966 and reinforced my long-standing russophilic feelings. I love the people and the culture and as I will point out later this feeling has never subsided. After our trip to Moscow and Leningrad we followed up with a week at a taxonomy conference in Prague. It was there that we hatched the idea of building an outreach organization, which we dubbed "Bergey's International Institute", which would house the book efforts and serve as a place to train an international cadre of taxonomists.

At the next meeting of the Board we presented a proposal to move the office to a new venue and begin to find funding for the Institute. We put out feelers to a number of institutions that might be willing to house the office and provide a professorship for me and my successors. After a period of negotiations and site visits we made an agreement with Michigan State University that was agreeable to both parties. The advantages of MSU were that they had a large, active microbiology department and also housed the NSF-funded Center for Microbial Ecology (CME), directed by the very capable James Tiedje, who was instrumental in facilitating the move. In December 1990 my wife and I and the Bergey office moved to East Lansing (Figure 2).

Before the move to Michigan the Trust was anxious to take on the task of compiling a 9th edition of the determinative manual. There were a number of potential difficulties standing in our way, not the least of which was the paucity of phenotypic information on many taxa, especially those newly described. The use of rRNA analyses to delineate new groupings was not followed by exhaustive phenotypic descriptions. In fact, a number of species were not cultured and, of course, the traditional method of differentiating bacteria was not possible. Noel Krieg took on the task of outlining content and the construction of the preliminary keys to the major groups. After much discussion we decided on a

Figure 2. Trustees of Bergey's Manual Trust at Michigan State University in 1991. Lower step, L to R: R.G.E. Murray, K.-H. Schleifer, J.G. Holt; upper step, L to R: N.R. Krieg, N. Pfennig, J.T. Staley, P.H.A. Sneath and D.J. Brenner.

format and, in conjunction with Williams & Wilkins, on the makeup of the book. I stuck to my firm belief that it had to be affordable and sturdy. It was put together rather quickly with the help of a large group of editors and authors, and was published in soft cover in 1991. There was one major hitch in its production. The publisher had contracted the copy-editing to a person not on their staff and who was not aware that the language in descriptions was in what I like to call "Bergeyese". This is the use of incomplete sentences: "cells elongated", "Gram-negative", and "chemoautotrophic". The person had begun to put all such phrases into complete sentences and as the first galleys came in I saw what was being done. I immediately called our editor, Bill Hensyl, berated him for not warning the copy-editor of our quirk, and insisted he convert the text back to Bergeyese. It was the start of the troubles we began to have with Williams & Wilkins, but more of that later.

Very significant changes occurred in my personal life at this time. My wife of 36 years, Bess-Gene, died suddenly of a heart attack in January 1992. This left a big void in my life; I was in a new town without family near or many friends outside the department. Everyone was very supportive and I kept myself busy trying to find money for the Institute, dealing with the publisher and teaching. My life changed again in 1993 when I met and later married Lydia Kalinina, a well-known protozoologist and cryobiologist from St Petersburg,

Russia, who was a Visiting Scientist at the CME.

My last book project for Bergey's was to compile a list of bacterial names, with the help, and co-authorship, of our two past and present secretaries, Cynthia Pease and Connie Williams, and a grad student, Mary Ann Bruns. It was a small book, part of a series for medical transcriptionists, entitled *Stedman's/Bergey's Bacteria Words*. It was a reworking of the index of names in the last edition.

As I mentioned above we began to have a troubled relationship with Williams & Wilkins. They had published all the Bergey books starting with the 1st edition in 1923. They had been a pleasure to work with, being a small family-owned specialty publisher of medical books. All the people at the company I had worked with, from the president on down, were very congenial and helpful. However, the company had become increasingly anxious about its "bottom line" and top management had changed from family members to MBA-trained professionals. Added to this were the increasing costs of producing the manuals. Royalties were no longer adequate to cover our expenses and we had to turn to the publisher for advances. There were many acrimonious discussions with Williams & Wilkins at our meetings and through the mail. About this time the company was sold to a big Dutch publisher and we knew we would have to change. The final arrangement was carried out by my successor in which Springer-Verlag became the publisher of the Bergey lineup. As an academic all my professional life I was not happy with my immersion in the unpleasant life of the business world.

One of my last tasks as editor was to begin constructing a phylogenetic outline classification of the prokaryotes based on the rapidly expanding knowledge gleaned from rRNA comparisons. The next edition of the systematic manual finally would be able to be organized phylogenetically. It was going to be necessary to have a complete listing of all the taxa so far described, and new ones that were being published frequently. I made the outline to the level of species and altered it when new names and combinations were validly published. It was also necessary to give names to the higher taxa, and I proposed tentative ones for all. Many of these names are finding their way into the current 2nd edition.

Figure 3. John G. Holt at the Trust meeting at its new home in Athens, GA, in 2007.

In 1995 I informed all concerned that I intended to retire on schedule at the end of the 1995–96 school year, and that the Board and department needed to find a successor who would take over my editorship and professor position. After an international search following university guidelines, the department, with Board of Trustees concurrence, chose George Garrity to be the new Editor-in-Chief and Professor. I continued to live in the MSU area, occasionally consulting with George, but never looking over his shoulder, until 2000 when I reached the mandatory retirement age for the Trust of 70.

In 2001 Lydia and I moved to Venice, Florida, where we are 5 miles from the Gulf of Mexico and out of the cold and snow. We spend most summers in our beloved St Petersburg, Russia, where Lydia has maintained her apartment, and sometimes we go to other parts of Europe.

Sometimes I wonder why I quit so early compared to my predecessors. Did I get burned out? Or just lazy? Or wanted to let younger people do it for a change? Probably all of the above. Whatever the reason I am so glad that Carl Woese and the others made taxonomy so appealing to so many young investigators, and I am happy to see them make such magnificent strides in our field. I wish the best for the Bergey family in their newest home at the University of Georgia (Figure 3), and may the *Manual* continue to appear in whatever form into which it evolves.

A life with some strange twists (mostly helical)

Noel R. Krieg

I was born on January 11, 1934 in the city of Waterbury, CT, the only child of Julius A. and Helen S. Krieg. I attended elementary school in Waterbury and, with my parents' help, learned to read at an earlier age than most children. In 1941we moved to the country, to the rural town of Middlebury, CT. Our home was surrounded by cow pastures, pine forests, and farm ponds, and it was a wonderful place in which to grow up and explore. I had few playmates other than those at school, but I had many activities to keep me busy—watercolor painting, making motorized gizmos with a Gilbert erector set, a dog that was my faithful companion, blueberry picking, etc. My beloved parents were always interested and supportive, and I had a very happy childhood.

My dad worked in the city at a large brass manufacturing company, where he introduced several innovative office methods. He was very creative and always had time to make clever things for me. For instance, one Christmas he constructed an illuminated, animated diorama from brass, oatmeal boxes, Christmas ribbon, and an electric clock motor. The diorama showed the Magi riding their camels toward the star of Bethlehem, and as the tiny camels travelled, their jointed legs moved exactly like real ones. (The intricate mechanism still works, even after 70 years, and I treasure it.)

My mother had studied at the Virgil Conservatory in New York and had been a concert pianist. After she married she gave up her career, although she still taught the piano. She often played concert pieces for me, especially Grieg and Liszt, and it is due to her that I developed a love of classical music and the arts.

When my mother tried to teach me to play the piano, I confess that I was not very diligent, being too busy looking at insects and pond water specimens with the toy Gilbert microscope that had been given to me one Christmas. (It was the only microscope I have ever known that had square lenses, each sandwiched between two circular diaphragms!). Its resolving power was poor, but money earned from the making and selling of shell jewelry, Christmas ornaments cut from tin cans, and plants potted in periwinkle shells, all enabled me to take a big step up—the purchase of a 75× microscope with achromatic lenses. My notebooks from 8th grade through high school are filled with observations on hydras, water fleas, and algae obtained from a delightful stagnant pond in a nearby pine forest. In my high school biology course, I often supplied such live specimens for the class. My favorite book, Animals Without Backbones by Ralph Buchsbaum, had wonderful stories and pictures. I made a photomicrographic apparatus by adapting an old bellows camera to my microscope and learned how to develop film, and a homemade enlarger allowed me to print the pictures (Figure 1). Eventually I earned enough to purchase a Bausch and Lomb 500× microscope. My parents encouraged me in all these activities.

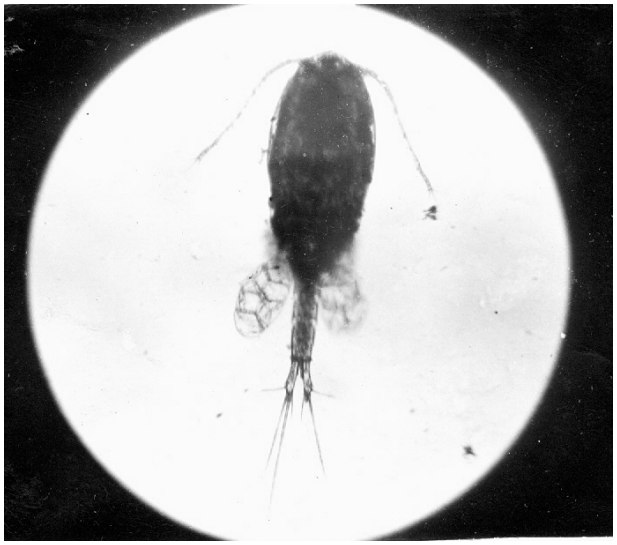

Figure 1. A cyclops, a pond water crustacean ranging from 0.5 to 2 mm in length, with empty egg sacs. This was the first picture taken with the homemade photomicrographic apparatus. (Photo courtesy of N. Krieg.)

While in high school, I constructed a microprojector from a microscope objective lens and a series of magnifying glasses. The light source—an arc lamp made from carbon rods extracted from old dry cell batteries—was bright enough to allow a 2-m-wide projection of live, motile daphnias, cyclops, and paramecia onto a wall, which ut-

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terly fascinated those who saw it. It easily outperformed the expensive Bausch and Lomb microprojector purchased by the high school for its biology program and earned me first prize at a regional science fair.

Then a new world opened up for me. I found a marvelous book – *Microbe Hunters* by Paul DeKruif – that introduced me to the romance and adventure of bacteriology. I am sure that many other microbiologists must have also been "turned on" by that glorious book. Then and there I decided to devote my life to bacteriology. After obtaining various peptones and something called "agar-agar", I began culturing bacteria in an incubator made from an old picnic ice-chest. An electrical engineer helped me construct a very accurate thermoregulator for the incubator, based on the expansion of ether vapor. I recall that one of my cultures made my parents very apprehensive, as it was purportedly a culture of *Mycobacterium tuberculosis*, which I had purchased from a biological supply house. Although acid-fast, the organisms could not actually have been tubercle bacilli, since they grew in only 48 h.

I entered the University of Connecticut in 1951, supported financially by my parents and by funds earned summers by working at a brass company and at a clock manufacturing company. I was disappointed to learn that, although the university offered a freshman course in microbiology, it was a terminal course for non-majors. I had to wait two very long years before I could take General Bacteriology, while first taking mundane subjects such as Organic Chemistry (which seemed to deal mainly with petroleum derivatives). Finally I could register for General Bacteriology, and it was my good fortune to have Professor Robert C. Cleverdon as the instructor. He was a dynamic teacher who did not stand on formality and would do almost anything to get you to understand and remember concepts. I will never forget his lecture on bacterial morphology. After saying that bacterial flagella could be many times the length of the cell, he drew a flagellum, and when he reached the end of the blackboard, he kept right on drawing it on the wall halfway around the room! In 1955–1957 as a master's student, I was fortunate to take Professor Cleverdon's course in Bacterial Physiology. It was there I learned that the amino acids and sugars so fleetingly mentioned in Organic Chemistry were not just powders in bottles; they were the stuff of life! I developed a passion for biochemistry and metabolic pathways, as well as learning the importance of distilled water and ultra-clean glassware in studying bacterial nutrition. Unfortunately, during a class experiment with a Warburg respiration apparatus, I managed to break a very expensive hand-blown manometer. I feared the worst as I slowly made my way to Dr Cleverdon's office, but when I shakily confessed what I had done, he merely looked at me, shrugged, and said, "Oh well, glass is made to break". After that, I would have done anything in the world for him! In an experiment for his Bacterial Physiology course he assigned us the project of doing a growth curve, but we could not use any of the common organisms, I chose an azotobacter, and the growth curve took 2 days and nights with hourly plate counts, direct counts, and nephelometer readings. We were all exhausted by the third day but were heartened by finding Professor Cleverdon cooking pancakes and eggs for our breakfast on the gas burner in the media prep room! That evening he invited us to his home for dinner, and we had a wonderful evening listening to his recordings of Anna Russell singing hilarious operatic sketches. Robert Cleverdon was a Renaissance man, having earned degrees in Music, German, and Bacteriology. During my subsequent years at Virginia Tech I have often seemed to sense his wonderful spirit guiding my teaching.

Figure 2. 1955 watercolor of bacteriology lab bench. (Painting by N. Krieg.)

The years at the University of Connecticut were golden ones, and I was extremely happy to be in such a notable bacteriology department. Professor Walter E. Kulp was the department head when I began my studies. He had worked with Leo F. Rettger at Yale in the 1920s, and so I felt a sense of continuity with the microbiologists of the past. I recall that on one snowy winter afternoon with the wind howling outside, I was in the lab painting a watercolor of my bench and microscope (Figure 2). Old Professor Kulp passed by, saw what I was doing, and remarked kindly that it was "good to be in the lab on a day like this".

An outstanding teacher, Stanley E. Wedberg, who had investigated the possible role of houseflies as vectors of poliovirus and who also had written an excellent introductory microbiology textbook called *Microbes and You*, directed my master's thesis. He suggested that I investigate the role

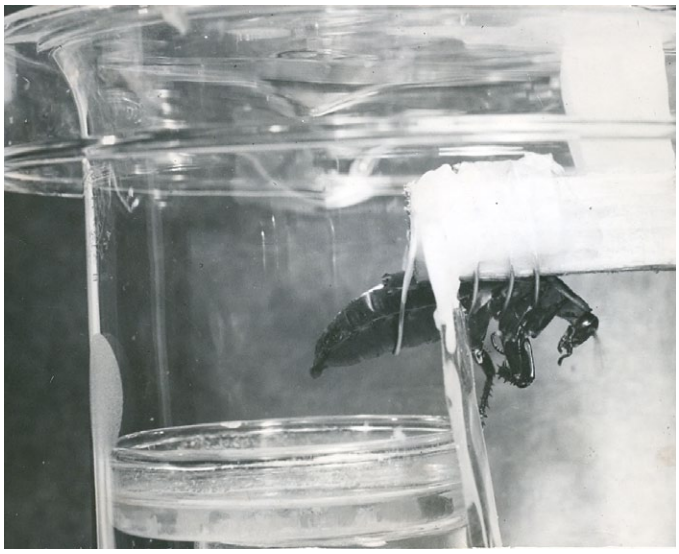

Figure 3. A *Blaberus* cockroach mounted under the lid of a large beaker so that the feces could be collected in a small Petri dish of *Salmonella* enrichment medium. (By permission, from Am. J. Trop. Med. Hyg. 1959, *8:* 119–123.)

of *Blaberus* cockroaches in spreading typhoid bacilli, as these insects could travel from sewers into kitchens (e.g., was the raisin bread in the pantry really raisin bread?) The large roaches were flown in from Cuba at $0.50 a head for the study. I found that after feeding billions of *Salmonella typhi* cells to each roach, the salmonellas completely disappeared within six hours. Examination of the entire alimentary tract failed to find any trace of them. However, *Salmonella enteritidis* did persist in the roach stools for 6 months (Figure 3). According to the literature, the pH of insect guts had only been measured colorimetrically with dyes, but by using a homemade microquinhydrone electrode that could accurately measure the pH in as little as 0.01 ml of fluid, I found that the crop of the insects had a pH of 4.5, which subsequent experiments showed to be lethal to *S. typhi* but not *S. enteritidis*. The acidity was due to acid production by certain bacteria that I isolated from the insects' crops (Krieg et al., 1959). In these experiments, the roaches had to be fed daily, and my parents were not exactly overjoyed when I brought them all home to keep them warm during Christmas vacation.

During this period, I remember being greatly impressed by the large *Bergey's Manual of Determinative Bacteriology* that sat enthroned in splendor on the departmental conference room table. It was regarded as almost sacred, containing the descriptions of all known genera and species of bacteria. Never did I dream that years later I would have the honor of editing such a publication!

My doctoral work at the University of Maryland began in 1957 under the direction of one of the wisest professors I have ever known, Michael J. Pelczar. He was the first to introduce me to the world of bacterial nutritional syntrophisms, and he asked me to analyze a nutritional mutualism between *Lactobacillus plantarum* and *Streptococcus* (now *Enterococcus*) *faecalis*. In a defined medium lacking phenylalanine and folic acid, neither organism grew separately, but the two grew well together, each providing a compound required by the other. The analysis involved bioautography and microbiological assays to identify the various compounds that were being interchanged between the two species (Krieg et al., 1960).

During my doctoral program at Maryland I had the pleasure of knowing some brilliant fellow students: Ronald J. Gibbons, Charles W. Griffin, David A. Power, Philip J. Provost, and Eddie C. S. Chan. I never imagined that in later years I would become a co-author of introductory microbiology textbooks with Eddie Chan and Professor Pelczar.

In 1960 at the age of 26, with a fresh PhD degree, I accepted a position as a microbiology teacher, viz., as an assistant professor at the Virginia Polytechnic Institute (now Virginia Tech) in Blacksburg. At first I held a joint appointment with the department of Biochemistry and Nutrition and the Department of Biology, but finally only with the latter. I have never regretted that decision. The Biology Department at Virginia Tech has always remained remarkably free of the empire building and infighting that plague some departments, thanks to wonderful colleagues and astute department heads, all of whom I hold in the highest regard.

When I first came to Blacksburg, I lived at the University Club. One day, in the club lounge, I looked up and was astonished to see the tall figure of Robert M. Smibert looming over me. Like me, Bob had earned a doctorate at the University of Maryland, but in Veterinary Microbiology, and thus we had moved in different worlds. I had no idea that he had recently joined the Veterinary Science Department at Virginia Tech. We became good friends and later synergistically shared the teaching of Pathogenic Bacteriology for many years. Bob was on most of my graduate students' advisory committees and I on his. Bob later became a key figure in the establishment of the VPI Anaerobic Laboratory, his specialty being the physiology of anaerobic spirochetes.

I spent my first two years at Virginia Tech teaching nine different bacteriology courses, some of which had never before been offered, such as undergraduate Bacterial Physiology and graduate Viruses and Rickettsias. Some relief was obtained when an outstanding dedicated teacher, Robert E. Benoit, joined the department in 1962. Together we estab-

lished a microbiology section in the department and saw it burgeon over the years.

Because of the initial heavy teaching load, I did not publish any articles until 1964, when my graduate student Roxanna Neikirk and I described the sensitivity of *Clostridium sporogenes* to sulfonamides in the absence of folic acid but not in its presence (Neikirk and Krieg, 1964). Later, my student Kay H. Culbert and I investigated several microbial syntrophisms in association with Henry Bungay and his student Adnan Shindala of the Civil Engineering Department. Henry's new Coulter electronic particle counter proved exceedingly useful in differentiating and enumerating the various organisms in the mixed cultures (Shindala et al., 1965).

After my first three years at Virginia Tech, I convinced my parents to move from the cold winters of Connecticut to the milder climate of Virginia. My Dad, who was now 73 and retired, had a house built in Blacksburg. I was happy that they now lived close to my apartment, so that I could take care of them if the need arose.

During the next 45 years at Virginia Tech, there were several different phases to my activities, and it seems best to treat each of these separately in the following sections.

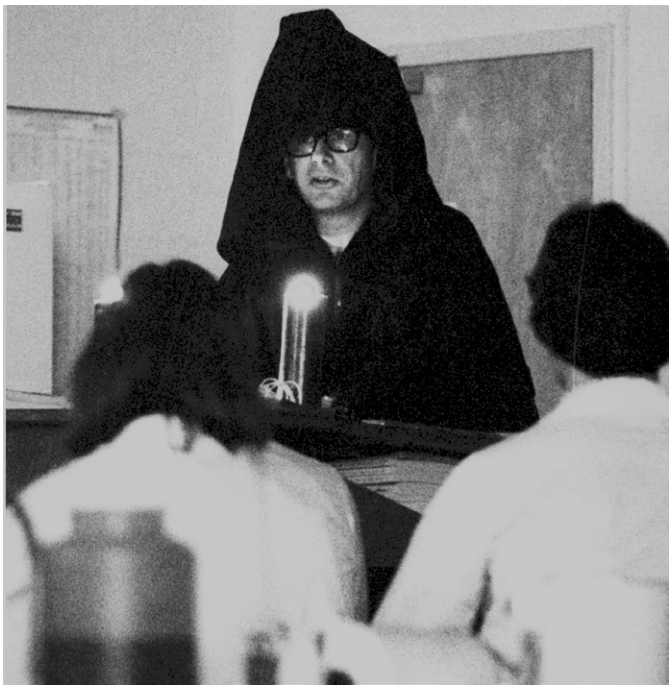

Figure 4. Professor Krieg attired as the "Spirit of Typhus" in 1980, explaining to a Pathogenic Bacteriology lab section how typhus and plague together changed the course of western history. A black veil and glowing eyes were later embellishments. (Photo courtesy of N. Krieg.)

Teaching

Because of the many GTAs I had during my masters and doctoral programs, I came to realize that I very much enjoyed teaching. My models were always Professors Cleverdon, Wedberg, and Pelczar. By example, they taught me eight principles of teaching, which I have always tried to follow:

1. It is essential to have a thorough knowledge of the subject.

2. An active research program enables a teacher to speak with some insight as to how science actually works.

3. Enthusiasm is a major factor in effective teaching. A teacher's enthusiasm can be contagious. And how could one not be enthusiastic about microbiology?

4. A teacher should impart a sense of drama, romance, or adventure about the subject. For instance, during many years of teaching Pathogenic Bacteriology, my laboratory sections were annually visited by the "Spirit of Typhus," who crept into the darkened classroom attired in medieval robes and hood, with black veil, glowing (battery-operated) eyes, and a cackling laugh (Figure 4). The Spirit regaled the class with some horrendous stories from Hans Zinsser's famous book, Rats, Lice and History, and it finished with a heartfelt wish for "the good old days" when it and plague wiped out a third of the population of Europe. It then proceeded to scatter some lice (rice grains, actually) toward the audience. I am told that the students who witnessed this event never forgot it. Professor Cleverdon would have been proud of me!

5. A teacher should provide many concrete examples as the pegs on which to hang abstract concepts, e.g., real case histories of the epidemiology of outbreaks as published in *Morbidity and Mortality Weekly Report* can help students better understand the principles of infection and immunity.

6. Most people think in terms of mental pictures, and thus good diagrams and figures are essential in helping students understand concepts.

7. Laboratory experiments must work properly, which can only be done by testing them thoroughly in advance.

8. Finally, teaching is an art, not a science, and varies with each teacher. What works for one may not work for another. I commend to the reader's attention *The Art of*

Teaching by Gilbert Highet (1950).

In regard to my teaching, I have never felt as astonished and humbled as when in 1978 the American Society for Microbiology notified me that I had been awarded the Carski Distinguished Teaching Award! I learned later that the committee had received a huge number of recommendations from my former students, to whom I will be forever grateful.

Spirillum volutans et al.

My interest in spirilla began in the early 1960s when a student in my introductory microbiology laboratory called me over to look at something strange in his wet mount of a hay infusion. I saw some huge helical organisms swim by like freight trains, and I had to switch to low power to observe them. After consulting *Bergey's Manual*, I concluded that the organism must be *Spirillum volutans*. One of its fascinating features was that, despite its large size, it had never been isolated. One reason was that it was vastly outnumbered in mixed cultures, so that dilution to extinction would not work. I discovered that in 1962 Sydney and Beatrice Rittenberg at UCLA had in fact found a way to isolate *S. volutans* from a mixed culture that had been maintained for years in their laboratory. Due to its high swimming speed, the organism was sometimes the first to reach the end of a long, flattened capillary tube before the contaminants did, whereupon the tube was broken behind it and the organism expelled into sterile broth. However, the isolated spirilla failed to grow alone in any medium and would grow only in sterile media contained in a dialysis sac suspended in a mixed culture of other bacteria. The latter were apparently producing a growth factor needed by the spirilla—a factor that could not be replaced by a great variety of media supplements. I gave my graduate student J. Scott Wells what I thought would be an easy project of isolating and then growing the organism without the dialysis sac.

After many attempts, Scott isolated a pure culture from hay infusion prepared with water from the Virginia Tech duck pond, using the Rittenbergs' dialysis sac method for maintaining the isolate. Like the Rittenbergs, we could not grow the isolated organisms free of the dialysis sac. One day, when Scott was looking at a wet mount of the organism, he was called away to the telephone. When he returned he was about to discard the wet mount, but he gave it one last look through the microscope. Then he called to me excitedly to come and look! The spirilla had congregated into a band of cells located about 0.2 mm from the edge of the wet mount. A few nonmotile cells were at the very edge or in the center of the mount, but, in the band, the cells were highly motile, swimming back and forth. The same thought occurred to

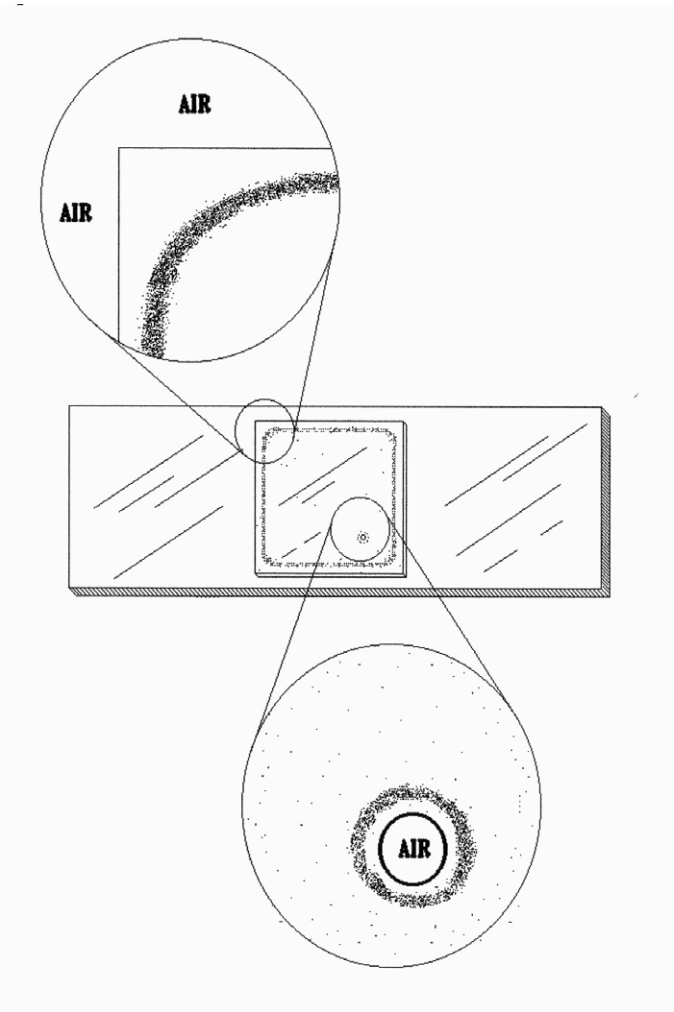

Figure 5. Microaerophiles such as *Spirillum volutans* form a band of motile cells near but not at the edge of the cover slip of a wet mount. Curvature of the band occurs at the corners, where O_2 is diffusing from both sides. Motile cells do not occur at the very edge of the mount (as would occur with an aerobe) because the high O_2 level is toxic. Few cells occur in the center of the mount because the cells' respiration has used up the O_2, creating anaerobic conditions. Microaerophiles will also form a band near a trapped air bubble. (Drawing by N. Krieg.)

both of us: the organism was a microaerophile! There was no mysterious growth factor provided by other bacteria; instead, they were merely using up some of the dissolved O_2 in the culture (Figure 5). We began pumping out jars to obtain various levels of O_2 and discovered that our spirillum could grow in ordinary nutrient broth, but only at O_2 levels from 1 to 9% (Wells and Krieg, 1965). Thus began my interest in microaerophiles—organisms for which O_2 is both a blessing (a terminal electron acceptor for respiration) and a curse (too much is toxic).

Potassium succinate improved growth of *S. volutans* enor-

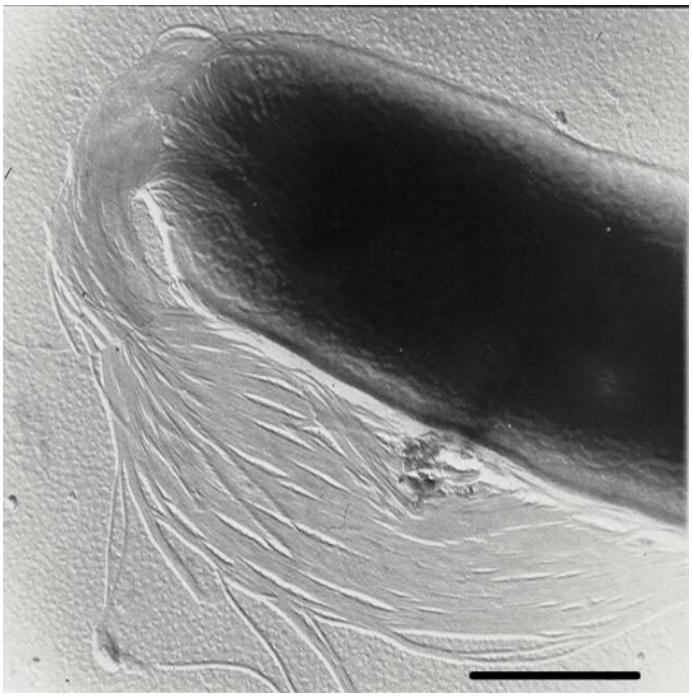

Figure 6. Electron micrograph of a flagellar fascicle of *Spirillum volutans*. Bar = 1 µm. (Photo by N. Krieg.)

mously, and we could even obtain colonies in semisolid media. My student B. H. Caraway and I discovered that the organism used O_2 as its only electron acceptor. The cells would migrate as a band aerotactically to self-created O_2 gradients along capillary tubes (Caraway and Krieg, 1974).

Bacteria are coccoid, rod-shaped, or helical, but the basis of bacterial shape is not yet fully understood. Although some progress has been made recently (e.g., the role of protein MreB in determining a rod shape), no one has yet explained satisfactorily what makes spirilla helical, or even what the function of helicity is. It certainly does not increase surface to volume ratio. Neither is it required for motility, since straight mutants of *S. volutans* can swim just as fast as the helical cells (Padgett et al., 1983).

Spirillum volutans has large flagellar fascicles at each pole (Figure 6), which form cones of revolution (Figure 7). When the cell reverses its motion, both fascicles simultaneously reverse their rotation and also the orientation of their flagellar fascicles (Figure 7, A and B). B.H. Caraway and I discovered that low levels of some compounds such as chloral hydrate and phenol caused uncoordination, with both fascicles becoming the "head" type, whereas other compounds such as $MgSO_4$, $NiSO_4$, and $CuSO_4$ caused both fascicles to become the "tail" type. Also, exposure of normal cells to pH 4.4 caused dual-tail uncoordination, while pH 9.9 caused dual-head orientation. Recoordination of cells was accomplished by washing cells free of agent, application of metal-complexing agents, or, in the case of uncoordination

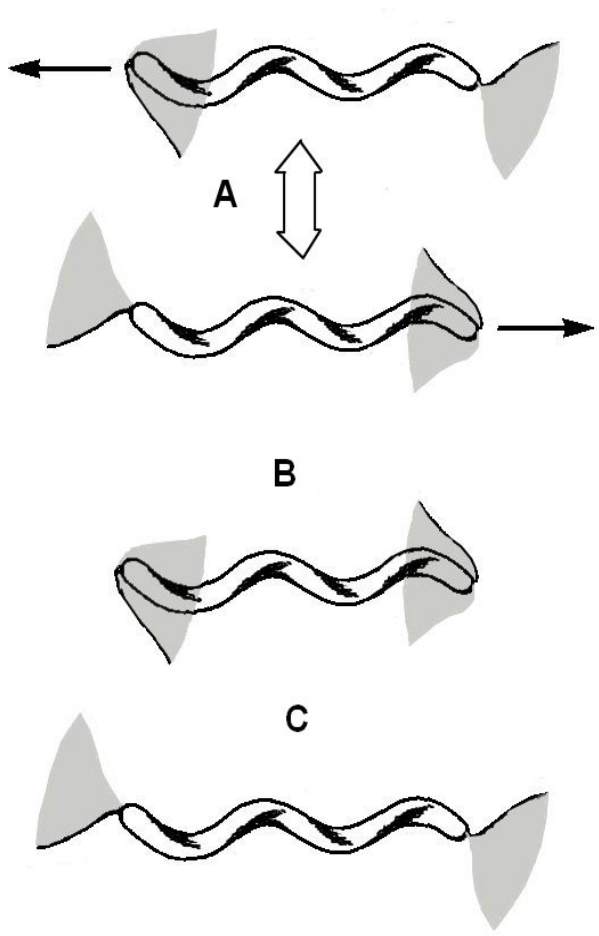

Figure 7. Diagram of *Spirillum volutans* showing orientation of the cones of revolution of the flagellar fascicles during normal back and forth swimming (A) and (B). Arrows indicate the swimming direction. Some chemicals cause "dual-head orientation" (C, top), whereas others cause "dual-tail orientation" (C, bottom); in either case, the cells cannot swim, due to the opposing propulsion. (Drawing by N. Krieg.)

by pH, readjustment of pH (Figure 7, B and C). After hearing a seminar on the use of the breathing behavior of fish as a biological monitoring agent for industrial pollutants about to be discharged into streams, it occurred to me that perhaps the uncoordination phenomenon in *S. volutans* might be useful in such monitoring, and it would be far easier than the fish method. This led Jean H. Bowdre and me to devise a simple method for doing this (Bowdre and Krieg, 1974).

In 1971, I asked my students Walter J. Strength and Bilquis Isani to study another unusual aquatic organism, which we named *Aquaspirillum* fasciculus. This strange bacterium exhibited bipolar flagellar fascicles clearly visible by dark-field microscopy. Considering that flagella were supposed to be fairly rigid structures, these fascicles exhibited an amazing variety of activities including helical wave propagation,

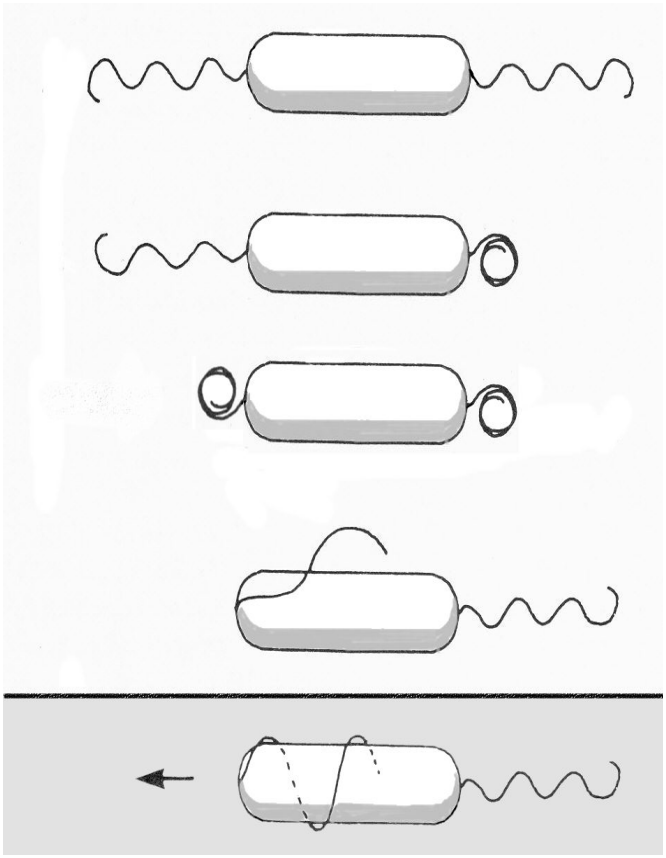

Figure 8. Behavior of the flagellar fascicles of *Prolinoborus fasciculus* in nonviscous media (top diagrams) and in viscous media (bottom diagram). The arrow indicates the direction of swimming. (Drawing by N. Krieg.)

basal bending, and an ability to coil up like springs (Figure 8), all of which were documented with cinephotomicrography. Despite the flagellar activity, the cells did not swim and merely "floundered about". However, when the viscosity of the medium was greatly increased, nearly every cell could swim freely and steadily in straight paths—just the opposite of what occurs with ordinary flagellated bacteria. As with *S. volutans*, the organism had a strictly respiratory metabolism and an inability to attack carbohydrates, and thus I thought it was probably a spirillum that had simply "forgotten" how to be helical. However, DNA–rRNA hybridization by Pot et al. (1992) showed that the organism was in fact not closely related to aquaspirilla, and it was placed in a new genus as *Prolinoborus fasciculus*. It is amazing that microbiologists have given little attention to the mechanism of its extraordinary flagellar behavior.

I encountered another unusual bacterium in 1968 while examining the drainage from an abandoned coalmine near Blacksburg. A pale yellow, fluffy material occurred in the first meter of the effluent, and microscopic examination revealed numerous twisted stalks characteristic of *Gallionella*. I added some formaldehyde to samples of the material to preserve the organism for class. Several weeks later, however, I noticed that the stalk material had greatly increased in these samples. Based on this resistance to formaldehyde, my student Jay W. Nunley and I developed a method for obtaining pure cultures of the organism (Nunley and Krieg, 1968). We maintained pure cultures of the organism by serial transfer for 14 months and in continuous flow culture for 3 months.

Taxonomic studies

The taxonomy of the genus *Spirillum* had not been studied since 1957, and I decided that my lab should do something to update it. My research group included my students Phillip B. Hylemon, Jean H. Bowdre, Scott Wells, together with Dr Thomas MacAdoo—a Greek and Latin scholar who was our revered nomenclature authority— and Holger Jannasch of the Woods Hole Oceanographic Institution, who had isolated several new marine spirilla. Probably the main reasons why the taxonomy of the aerobic spirilla had been neglected was that the spirilla are difficult to isolate and are largely "inert"; i.e., most of the usual characterization tests that are so useful for the *Enterobacteriaceae* are negative for spirilla. Thus we had to rely on other phenotypic characteristics, such as wavelength and diameter of the cell helix, pigment production from aromatic amino acids, tolerance to NaCl, and especially the testing of 49 potential sole carbon sources and 26 sole nitrogen sources. Our study included all of the available strains and employed a uniform methodology to provide a valid basis for comparison. In the 1970s, the importance of DNA base composition in taxonomy was becoming recognized; therefore, we determined the mol% G+C of the DNA of each strain. Based on the phenotypic features and G+C values, we divided the genus into three genera, with the original name *Spirillum* being restricted to large microaerophilic freshwater forms having a mol% G+C of 36–38; this genus contained only *S. volutans*. We proposed the name *Aquaspirillum* for the aerobic, freshwater forms having a mol% G+C of 49–65, and the name *Oceanospirillum* for the aerobic, marine forms that did not attack carbohydrates and had a mol% G +C of 42–48 (Hylemon et al., 1973).

This arrangement was simple and useful, but the use of DNA–DNA hybridization, rRNA–DNA hybridization, and rRNA gene sequencing by other taxonomists led to the realization that nature does not always follow our human-made schemes, as practical as they may be. For instance, we had named a new spirillum *Aquaspirillum bengal* because it had been isolated from a freshwater pond in West Bengal. It was similar in many ways (including the mol% G+C of its DNA) to *Aquaspirillum serpens* and *Aquaspirillum putridiconchy-*

lium, but differed by its high optimum growth temperature (41°C), formation of water-soluble pigments from tyrosine and tryptophan, and several other nutritional, biochemical, and serological characteristics. However, in 1985, Boivin et al. found that the organism exhibited high DNA–DNA hybridization with *A. serpens*, and they reclassified it in that species.

In fact, in phylogenetic terms, the genera *Aquaspirillum* and *Oceanospirillum* eventually were found by other taxonomists to be heterogeneous, and the various species have since been widely scattered among the *Proteobacteria*. Most of the original species of the genus *Aquaspirillum* have now been assigned to other genera (*Comamonas*, *Curvibacter*, *Giesbergeria*, *Herbaspirillum*, *Hylemonella*, *Insolitispirillum*, *Microvirgula*, *Novispirillum*, and *Simplicispira*), with only *A. polymorphum*, *A. putridiconchylium*, and the type species *A. serpens* remaining. Of the original species In *Oceanospirillum*, only *O. beijerinckii*, *O. maris*, and the type species *O. linum* remain, the others being assigned to *Marinospirillum* and *Pseudospirillum*.

An interesting taxonomic puzzle arose when my student Daniel Linn and I discovered that the characteristics of the ATCC type strain of "*Spirillum lunatum*" did not fit the original description of the species. A culture of what was purportedly the same strain from the NCMB seemed to consist of two kinds of organisms—many short vibrioid cells (resembling those in the ATTC strain) and a few larger spirillum-like cells. The paucity of the spirillum-like cells meant they could not be isolated directly by plating methods or by dilution to extinction. Consequently, we immunized a rabbit with the vibrioid cells and then added the resulting antiserum to the mixed culture. This caused the vibrioid cells to agglutinate and settle out, leaving a predominance of the spirilla forms in the supernatant, which then allowed their isolation. The vibrioid organism had single flagella, grew in the presence or absence of sea water, catabolized sugars, and had a DNA base composition of 63–64 mol% G+C, whereas the spirillum-like organisms had bipolar tufts of flagella, required sea water for growth, could not catabolize sugars, and had a DNA base composition of 45 mol% G+C. We did not identify the vibrioid organisms, but the spirillum-like organisms seemed to be typical oceanospirilla, and we named them *Oceanospirillum maris* (Linnn and Krieg, 1978).

During these taxonomic studies, I learned a great deal about the physiology of spirilla. For instance, for their initial enrichment it was essential to use serial transfers into media having very low levels of carbon sources to avoid overgrowth by other organisms. Moreover, carbohydrates

Noel Krieg in 1982.

were seldom used, and when they were, they were often catabolized by unusual mechanisms. For instance, my student Phillip B. Hylemon discovered that *Spirillum itersonii* (now *Novispirillum itersonii*) was impermeable to glucose, despite the occurrence of high levels of glucokinase activity in the cells. Oddly, the organism could take up fructose by a carrier-mediated transport system that was induced by either fructose or glucose – a system that was later discovered by others to be a fructose-specific phosphoenolpyruvate phosphotransferase system. Another organism *Aquaspirillum gracile* (now *Hylemonella gracilis*), formed acid from only D-glucose, D-galactose, and L-arabinose, and my student Barbara E. Laughon found enzyme activities characteristic of the Entner–Doudoroff and Embden–Meyerhof–Parnas pathways, but not the hexose monophosphate pathway.

I also found that certain marine spirilla undergo a mass conversion of the vegetative cells to a coccoid shape after the exponential phase of growth. Several other bacteria, including some pathogens, undergo a similar conversion. These coccoid bodies have been termed "viable but nonculturable" (VBNC). The "nonculturable" aspect was based on the inability of the cells to grow in fresh media, and the "viable" aspect was based, among other things, on the ability of the altered cells to fluoresce when treated with acridine orange (AO) and, in the pathogenic species, to be "resuscitated" by feeding young laboratory animals or by other means, although the validity of such resuscitation has been hotly debated. In the 1990s, my laboratory studied the VBNC phenomenon in *Prolinoborus fasciculus* (Koechlein and Krieg, 1998). Although the coccoid form did fluoresce with AO, agarose gel electrophoresis revealed that extensive rRNA degradation had occurred during their formation. The results supported the idea that the coccoid forms were degen-

erative forms rather than part of a life cycle. My laboratory also isolated and purified an autolysin from *Campylobacter upsaliensis* that might be involved in coccoid body formation (Santiwatanakul and Krieg, 1999).

My taxonomic studies of "*Spirillum lipoferum*" began in 1976 when the distinguished Brazilian soil bacteriologist Johanna Döbereiner appeared at my office. She had discovered a microaerophilic, root-associated, N_2–fixing, curved rod that was similar to an organism that had been described by Beijerinck in 1921–1922. Johanna had read the review by Hylemon and Krieg on the taxonomy of spirilla, and she asked me to find out if *S. lipoferum* was an *Aquaspirillum* species and, if not, to classify it appropriately. Johanna was a very dynamic and persuasive person, and I soon found myself agreeing to look into the matter. Our association turned out to be mutually beneficial. She was invariably supportive of my efforts, and I enjoyed working with her more than with any other scientist I have encountered.

I soon decided on phenotypic grounds and DNA base composition that *S. lipoferum* was not an *Aquaspirillum* species, a conclusion supported much later by rDNA sequence analysis (Fani et al., 2006). The main problem, however, was how many species did the 61strains that Johanna sent me represent. Was there a single species that was associated with many kinds of plants? Were there many species, each specific for a particular kind of plant? I decided to handle the problem by the best method available, viz., DNA–DNA hybridization, which my lab had never done before. Thanks to John L. Johnson of the Virginia Tech Anaerobe Laboratory, my graduate student Jeffrey J. Tarrand and I learned the procedure. The results indicated that two species existed, which we named *Azospirillum brasilense* and *Azospirillum lipoferum* (Tarrand et al., 1978). The latter was distinguished from the former by its ability to use glucose as a sole carbon source for growth in nitrogen-free medium, by its production of an acidic reaction in a peptone-based glucose medium, and by its requirement for biotin.

I had seldom looked at old cultures of azospirilla, but after publishing the *Azospirillum* article, I happened to do a Gram stain of some 48-h-old agar slant cultures. Most of the cells were rod-shaped or vibrioid and stained Gram-negative, but to my horror I noticed a few that were larger, ovoid, and stained Gram-positive. I never found these cells in young cultures. I thought, "Oh my gosh! Have I described a new genus and two species on the basis of contaminated cultures?" I frantically made streak plate after streak plate to isolate the Gram-positive form, but every colony gave rise to a culture that exhibited a few of them. Finally in desperation I asked Robert H. Gherna, the extremely knowledgeable

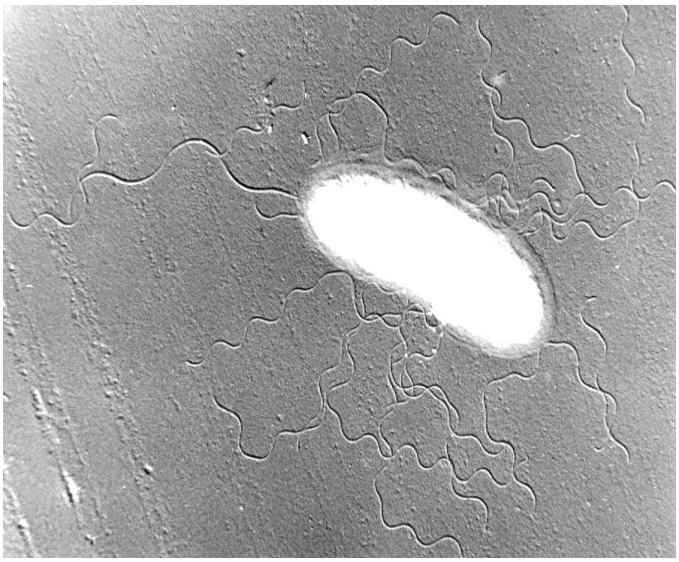

Figure 9. Mixed flagellation in *Azospirillum lipoferum*. (By permission, from Tarrand et al., 1978, Can. J. Microbiol. *24:* 967–980.)

curator of bacteria at the American Type Culture Collection (ATCC), if he thought that my cultures were contaminated. Bob examined them and told me not to worry, that he had seen such forms before in various other bacteria, and that the Gram-positive forms were cystlike forms that had developed a thick layer of condensed exopoplysaccharide on their surface. The thick layer mimicked the thick cell wall of a Gram-positive organism and thus it was responsible for the Gram-positive staining reaction. I heaved a great sigh of relief upon hearing this! His conclusion was later confirmed by electron microscopy of thin sections (e.g., Berg et al., 1980), and the cystlike forms are now known to impart desiccation-resistance to azospirilla (Lamm and Neyra, 1981).

I discovered still another peculiarity of azospirilla, in that the cells, which produced a single polar flagellum when grown in broth, also formed numerous lateral flagella of thinner diameter and shorter wavelength when grown on solid media (Figure 9). By chemical mutagenesis, my graduate student Patrick G. Hall and I obtained mutants having only a polar flagellum or only lateral flagella (Hall and Krieg, 1983). The mutant with only a polar flagellum could swim in broth but was unable to spread on semisolid (0.75% agar) media, whereas the mutant with lateral flagella could spread on semisolid media but could not swim in broth. We prepared specific antibodies for each type of flagella, and with an indirect immunoperoxidase technique we showed that the two kinds of flagella differed antigenically (Hall and Krieg, 1984).

My student Edwin M. Goebel and I found another puzzling

feature in azospirilla. *A. brasilense* could not use glucose as a sole carbon source, even though it had a complete Embden–Meyerhof–Parnas (EMP) pathway. We found the organism to be nearly impermeable to glucose; however, it could use fructose. We showed that it transported fructose by a phosphoenolpyruvate-phosphotransferase system, thereby forming fructose-1-phosphate. The latter compound then entered the EMP pathway by being phosphorylated to fructose 1,6 diphosphate (Goebel and Krieg, 1984).

In 1983 a new species, *Azospirillum amazonense*, had been described in Brazil, and my student Eileen C. Falk found that, although the strains of *A. amazonense* were related to one another at a DNA hybridization level of >57%, they showed no significant hybridization with the other two species of *Azospirillum*, indicating that they were a distinct species. But to what genus did *A. amazonense* really belong? At this time, the technique of rRNA–DNA hybridization was being used to explore broader relationships than could be revealed by DNA–DNA hybridization. Again, John Johnson showed us how to do this new technique, and with it Eileen found 90–96% rRNA–DNA hybridization between *A. lipoferum* and *A. brasilense* and 64–70% between these two species and *A. amazonense*. Thus, *A. amazonense* was indeed a member of the genus *Azospirillum*. Another putative *Azospirillum* species that had been described in 1984, "*Azospirillum seropedicae*", showed little rRNA–DNA hybridization with other *Azospirillum* species and Eileen and I recommended that it should not be included in the genus. (It has since been reclassified as *Herbaspirillum seropedicae*.)

We encountered an interesting taxonomic puzzle in 1983. Australian workers had proposed a new genus, *Conglomeromonas*, containing one species, *Conglomeromonas largomobilis*, and two subspecies, *Conglomeromonas largomobilis* subsp. *largomobilis* and *Conglomeromonas largomobilis* subsp. *parooensis*. Eileen Falk and I thought that the features of C. *largomobilis* subsp. *largomobilis* greatly resembled those of azospirilla (including its mixed flagellation). In fact, our rRNA–DNA hybridization studies showed that it did belong to the genus *Azospirillum* and, by DNA–DNA hybridization, was related to *A. lipoferum*; moreover, we found this organism to be a microaerophilic N_2 fixer. Other researchers have named the organism *Azospirillum* largimobile. In regard to the other subspecies, *C. largomobilis* subsp. *parooensis*, our DNA–DNA studies indicated that it was not related to *C. largomobilis*, *Azospirillum lipoferum*, or any other species tested. It is now known as *Skermanella parooensis*.

My colleague Robert Smibert was working with campylobacters and convinced me that, like spirilla, they needed a better classification. He suggested that we start with some microaerophilic strains called *Campylobacter sputorum* subsp. *mucosalis* that had been isolated from lesions of porcine intestinal adenomatosis. However, they differed phenotypically in a few respects from *C. sputorum*, and the DNA base composition was slightly different. My student Roy Martin (Marty) Roop performed some elegant DNA–DNA hybridization experiments and discovered that, although high levels of DNA hybridization occurred between all of the *C. sputorum* subsp. *mucosalis* strains tested, no significant hybridization occurred with any of the other *Campylobacter* species tested, including *C. sputorum*. Consequently, we reclassified *C. sputorum* subsp. *mucosalis* as a separate species, *Campylobacter* mucosalis (Roop et al., 1985).

In 1988, my student Louis M. Thompson III performed a comprehensive study of the phylogenetic relationships of all of the species in the genus *Campylobacter*, *Wolinella succinogenes*, and other Gram-negative bacteria by comparison of their partial 16S rRNA gene sequences as obtained by using the reverse transcriptase method (Thompson et al., 1988). This method has of course now been superseded by PCR methodology, but it was useful at the time. The results of our study showed that the campylobacters made up three separate rRNA groups, which we considered to represent separate genera. Moreover, the three groups were only distantly related to the alpha, beta, and gamma branches of the *Proteobacteria*, and they have since been reclassified in the class *Epsilonproteobacteria*. Group I contained the "true" *Campylobacter* species: *Campylobacter fetus* (type species), *Campylobacter coli*, *C. jejuni*, *Campylobacter laridis* (now *C. lari*), *Campylobacter hyointestinalis*, *Campylobacter concisus*, *C. mucosalis*, *C. sputorum*, and *Campylobacter upsaliensis*. Group II contained "*Campylobacter cinaedi*," "*Campylobacter fennelliae*," and *Campylobacter pylori* (all three are now classified in the genus *Helicobacter*), and *W. succinogenes* (which is now the sole species left in the genus *Wolinella*). RNA group III contained *Campylobacter cryaerophila* and *Campylobacter nitrofigilis* (both now in the genus *Arcobacter*).

It is important to correlate phenotypic characteristics with phylogenetic relationships, and our RNA group II was particularly interesting because one feature that seemed to be unique to *Helicobacter pylori* was the occurrence of sheathed flagella—a type of flagellation that is uncommon among bacteria and thus might have taxonomic significance. Because of the relatedness of C. *pylori* to *C. cinaedi*, *C. fennelliae*, and *W. succinogenes*, my student Yeong-Hwan Han and I thought that these latter species might also possess sheathed flagella. *Campylobacter cinaedi* and *C. fennelliae* did indeed exhibit sheathed flagella (Han et al., 1989).

Although *W. succinogenes* did not have sheathed flagella, the fact that three of the four species of rRNA group II had sheathed flagella and that none of the members of rRNA groups I and III had sheathed flagella was taxonomically significant.

An intriguing problem was the finding of anaerobes and microaerophiles in the same rRNA group, since anaerobes do not respire with O_2. Our rRNA group II contained the microaerophiles *H. pylori*, *C. cinaedi*, and *C. fennelliae*, but it also included the putative anaerobe *W. succinogenes*. Because *W. succinogenes* is oxidase-positive, a characteristic usually associated with organisms that can respire with O_2, this raised the question of whether *W. succinogenes* was truly an anaerobe. Although it does grow anaerobically by using fumarate as a terminal electron acceptor, it can use O_2 as a terminal electron acceptor under microaerobic conditions (Jacobs and Wolin, 1963). *Wolinella recta*, *Wolinella curva*, *Bacteroides ureolyticus*, and *B. gracilis* were also putative anaerobes, but Paster and Dewhirst (1988) reported their genetic placement was with the campylobacters. The fact that three of these species (*W. recta*, *W. curva*, and *B. ureolyticus*) were oxidase-positive suggested to us that they, like *W. succinogenes*, might actually be microaerophiles. My student Yeong-Hwan Han showed that the type strains of these species did in fact exhibit O_2-dependent microaerophilic growth with H_2 as the electron donor, with optimal O_2 levels ranging from 2 to 14% (Han et al., 1992). No growth occurred under 21% O_2, and scant or no growth occurred under anaerobic conditions (unless fumarate or nitrate was provided as an electron acceptor). Moreover, cyanide inhibited their uptake of O_2. We later showed that *W. curva*, *W. recta*, *B. ureolyticus*, and *B. gracilis* contained a membrane-bound cytochrome *b*, cytochrome *c*, and CO-binding cytochrome *c* (Han et al., 1992). Although *B. gracilis* was oxidase-negative, it also possessed cytochrome *c*. Proton efflux from anaerobic cells occurred upon addition of a pulse of O_2 and this efflux was inhibited by a protonophore, confirming that the organisms were indeed capable of respiring with O_2. *Wolinella curva*, *W. recta*, and *B. gracilis* are presently classified as *Campylobacter* species (Vandamme et al., 1991, 1995). The retention of the genus name *Bacteroides* for *B. ureolyticus* is unfortunate, as it implies that this organism is an anaerobe.

Oxygen toxicity

In the course of developing a defined growth medium (DGM) for *Spirillum volutans*, my graduate students Jean H. Bowdre and Paul S. Hoffman found that minute levels (10^{-5} to 10^{-6} M) of nor-epinephrine allowed *S. volutans* to grow under an air atmosphere (21% O_2) in broth (but not on agar). Epinephrine and 3,4-dihydroxyphenylalanine) were equally effective, and the configuration of these compounds (D, L, or DL) made no difference. Like *S. volutans*, the vibrioid microaerophile *Campylobacter jejuni* could normally use only low levels of O_2 as a terminal electron acceptor; however, unlike *S. volutans*, *C. jejuni* could grow on agar plates. Our strain of *C. jejuni* normally could not grow on *Brucella* agar under 17 or 21% O_2, but it could grow well under 6% O_2. Nevertheless, it grew well at 17% O_2 and moderately well at 21% O_2 if 2×10^{-4} M nor-epinephrine was added to the medium. Dihydroxyphenylalanine and protocatechuate were also effective (Bowdre et al., 1976). Our later studies showed that the ferrated forms of these dihydroxyphenyl compounds had the ability to destroy toxic forms of O_2 in culture media, to which microaerophiles are extraordinarily sensitive.

The microaerophilic nature of *C. jejuni* complicates its recovery from clinical specimens. In 1978, my students Hugh A. George and Paul Hoffman found that when *Brucella* agar was supplemented with ferrous sulfate, sodium metabisulfite, and sodium pyruvate (FBP supplement), the O_2 tolerance of the organism was greatly enhanced; moreover, growth responses occurred 1–2 days earlier than usual (George et al., 1978). The FBP supplement had the ability to destroy toxic forms of O_2 in the medium. Eventually the supplement came into wide use for culturing *C. jejuni* in clinical laboratories.

In 1979, Paul Hoffman, Hugh George, Bob Smibert and I showed that the addition of bovine superoxide dismutase (SOD) to *Brucella* broth or *Brucella* agar greatly enhanced the oxygen tolerance of *C. jejuni*. Catalase also increased oxygen tolerance. These enzymes unquestionably had to act externally to the bacteria. In fact, all of the diverse compounds that enhanced oxygen tolerance of *C. jejuni* shared the ability to quench either superoxide anions (O_2^-) or hydrogen peroxide (H_2O_2). On the basis of these and other data, we proposed that *C. jejuni* was more sensitive to exogenous O_2^- and H_2O_2 than are aerotolerant bacteria, despite the occurrence of SOD and catalase activities in *C. jejuni*. We proposed that compounds that enhance oxygen tolerance in *C. jejuni* acted by quenching O_2^- and H_2O_2 that occur spontaneously in these culture media.

I often told my graduate students to always write everything they did in their notebook, even the time of day they sharpened their pencils, but the students probably thought this was merely a whim of mine. However, the value of keeping a complete laboratory notebook became apparent when one student encountered some highly erratic results while doing spread plate counts of *C. jejuni*. The counts seemed to vary widely at the same dilution of inoculum, from several hundred colonies per plate to few or none. The student couldn't

understand what was causing these erratic results, because he had always prepared the inocula for the plates in exactly the same way. I asked him to examine his notebook and see if anything at all in his methodology could be correlated with his results. After going over everything, he told me that the only difference was that, when the low counts were obtained, he had prepared the medium in the afternoon, whereas when the high counts were obtained, he had prepared it in the evening. It occurred to me that during afternoons the lab was brightly illuminated by the many windows, whereas in the evening only relatively dim fluorescent ceiling lamps supplied the illumination. Experimentation showed that strong illumination in the presence of air did indeed render the culture media highly inhibitory, to the point where *C. jejuni* would not even grow at 6% O_2. Similar illumination under anaerobic conditions had no effect. Also, if the media were supplemented with SOD, catalase, or FBP-supplement, the inhibitory effect of light was greatly decreased. We soon learned that, to obtain consistent plate counts of *C. jejuni*, it was necessary to prepare the media freshly under either dim light or under red light (blue light was inhibitory), store the plates in the dark, and use the plates within 24 h. Such findings sometimes make microbiology seem more of an art than a science!

We wanted to see if the effect of illumination on media for *C. jejuni* might also apply to *S. volutans*. In 1982, my students Penelope J. Padgett and William H. Cover discovered that subjecting the media to light (unless it was red light) did indeed render the medium inhibitory to growth of *S. volutans* by generating H_2O_2. A combination of catalase and SOD prevented this inhibition. In fact, we could actually grow *S. volutans* on solid media for the first time, provided that we used a medium containing the FBP supplement, catalase, or SOD, and provided that the plates were protected from illumination and incubated in a highly humid atmosphere under low O_2 levels. Later, my student Scott Alban and I developed an improved method by which colony counts of *S. volutans* approached those by obtained by direct microscopic counts (Alban and Krieg, 1996).

Delving further into the strange effects of culture media components on growth, in 1994 my gradate student Jeffrey Hodge and I found that tolerance of *C. jejuni* to O_2 varied with different brands of the complex media used for plating. With some tryptones, growth occurred at 21% O_2, whereas with others there was no growth at 15% O_2 or higher. Consequently, we devised a chemically defined, agar-solidified plating medium to estimate the tolerance to O_2 of *C. jejuni*, *Campylobacter coli*, and *Campylobacter fetus* subsp. *fetus*, and also to assess the effect of added scavengers of reactive oxygen intermediates on O_2 tolerance. Several compounds such as allopurinol, azelaic acid, caffeine, cimetidine, TEMPOL and pyruvate markedly enhanced O_2 tolerance.

In a continuing search for reasons why campylobacters were inhibited by normal O_2 levels, my graduate student James A. Daucher and I considered the role of enzyme pyruvate: ferredoxin oxidoreductase. The way in which aerobes oxidatively decarboxylate pyruvate differs from the way used by anaerobes. Aerobes use a NAD^+-linked pyruvate dehydrogenase multienzyme complex that catalyzes the following overall reaction:

$$\text{Pyruvate} + NAD^+ + \text{Coenzyme A} \rightarrow \text{Acetyl-CoA} + NADH + H^+ + CO_2$$

Anaerobes oxidize pyruvate by means of a pyruvate: ferredoxin oxidoreductase (PFOR), which catalyzes the following overall reaction:

$$\text{Pyruvate} + \text{Coenzyme A} + 2Fd(ox) \rightarrow \text{Acetyl-CoA} + CO_2 + 2Fe(red) + 2H^+$$

Since microaerophiles are neither anaerobes nor aerobes but do respire with O_2, we wondered which of these enzyme systems operated in them. Accordingly, we tested 12 strains representing 11 *Campylobacter* species by two methods to see whether PFOR activity was present. We detected it in all of these species. Moreover, all strains were inhibited by metronidazole, whose inhibitory action is initiated by reduction of its nitro group by Fd(red). The results suggested that PFOR was a general characteristic of the genus *Campylobacter*, and since PFOR is oxygen-labile, this might also help to explain the O_2 sensitivity of these organisms.

My student Ross Zirkle and I developed a method for estimating H_2O_2-caused DNA strand breakage in intact cells and the subsequent repair of that damage, based on alkaline gel electrophoresis (Zirkle and Krieg, 1996). The method worked well and is still being used by some researchers. We would have liked to use the method with microaerophiles, but we thought it would be best to test the method with isogenic strains of *Escherichia coli* whose deficiency in DNA repair enzymes had already been well documented.

In 1986, peroxidases were implicated in the O_2 tolerance of *S. volutans* when my student Penelope J. Padgett obtained a variant of *S. volutans* capable of growth under an air atmosphere (21% oxygen) by sequential selection and stabilization of colonies. Both the variant and the wild-type lacked catalase activity, and they had the same levels of SOD activity. However, the mutant possessed 13 times more donor: hydrogen-peroxide oxidoreductase (peroxidase) activity than the wild-type. We also isolated an aerotolerant mutant

in one step by chemical mutagenesis followed by incubation under 21% O_2; this mutant had ca. three times the peroxidase activity of the wild-type.

In the late 1990s my student P. Scott Alban and I quantified the sensitivity of *S. volutans* to H_2O_2. We found the organism to be killed rapidly by H_2O_2 levels greater than 10 μM (Alban and Krieg, 1998). By chemical mutagenesis, we isolated a mutant that was able to survive and grow after exposure to 40 μM H_2O_2 and was also able to eliminate H_2O_2 added to the medium. The only apparent phenotypic difference between the wild-type and the mutant was that the mutant had high NADH peroxidase activity, whereas the wild-type had no detectable activity. NADH peroxidase had not previously been reported in Gram-negative bacteria or in bacteria having a strictly respiratory type of metabolism. One- and two-dimensional electrophoresis showed that the mutant constitutively expressed a protein that was undetectable and non-inducible in the wild-type. We cloned the gene that encoded the protein by using amino acid sequence data obtained by both mass spectrometry and NH_2-terminal sequencing. The sequence of the gene indicated a close relatedness of the protein to rubrerythrin and nigerythrin. Since no other proteins could be detected that were uniquely expressed in the mutant, it seemed likely that the novel protein played a key role in protection of the cells from peroxide. Our report was the first report of a rubrerythrin/nigerythrin-like protein occurring in an O_2–respiring organism, as previous reports of rubrerythrin or nigerythrin had been limited to anaerobic bacteria. As with PFOR, microaerophiles seem to have some features characteristic of anaerobes and other features characteristic of aerobes. In 1999, other investigators showed that rubrerythrin from anaerobes had NADH peroxidase activity – a result that was consistent with the phenotype of our H_2O_2-resistant strain of *S. volutans* (Coulter et al., 1999).

It is unlikely that further studies will be done with *S. volutans*. I had preserved many vials of the organism in a tank of liquid N_2, but when I retired, other faculty needed that tank. This did not worry me, because I knew that the type strain was preserved safely at the American Type Culture Collection (ATCC). However, when a colleague in Germany requested a culture of the organism recently, I discovered that the ATCC had lost not only my strain of *S. volutans* but also the Rittenbergs' strain. A different organism, *Spirillum* winogradskyi, has now been designated the type strain (Podkopaeva et al., 2009).

Bergey's Manual

In 1976 Professor Dr Robert G. E. Murray, the Chairman of the Bergey's Manual Trust, invited me to join the Board of Trustees, presumably because of my taxonomic work with spirilla. I did accept, but with some apprehension, because I felt that I was not a sufficiently eminent person to belong to that august body. However, the Board needed someone who was at least somewhat familiar with the Gram-negatives, there being a need for that particular area of expertise. Little did I know what would be in store for me!

It has been my good fortune to know my fellow board members as good friends and as brilliant scientists who were dedicated to bacterial systematics. By his wit and wisdom, Bob Murray channeled our discussions in productive ways, and he was a driving force to make the *Manual* a truly international publication rather than a largely "American" one. One way to do this was to have the Board meetings outside of North America every other year. Another way was to have a Board of international composition. In 1976 the Board consisted of Bob Murray in Canada, Stephen Lapage in England, Hans Lautrop in Denmark, and Marvin Bryant, James Staley, John (Jack) Holt, and myself from the United States. Jack Holt ably served the position of Editor-in-Chief of the Bergey publications until 1996. I refer readers to the excellent history of *Bergey's Manual* by Murray and Holt (2005).

From 1976 to 1979 I and the other Trust members were concerned about the next edition. It had been 17 years between the 7th and 8th editions and no one wanted such a long interval again. Hans Lautrop introduced the idea of expanding the scope of the *Manual* so that it would no longer be purely determinative but would cover all three areas of systematic bacteriology—classification, nomenclature, and identification. Thus we decided to include all information about the characteristics of the organisms—morphological, physiological, antigenic, ecological, and genetic—as well as their enrichment, isolation, and maintenance. To reflect this broadened scope, we decided to name the new publication *Bergey's Manual of Systematic Bacteriology* (BMSB), although we derived the 9th edition of *Bergey's Manual of Determinative Bacteriology* from its differentiation tables. Although we recognized that the molecular biological aspects of bacterial classification, such as 16S rRNA oligonucleotide cataloging, were extremely important for bacterial phylogeny, the data were still fragmentary. Consequently, we thought that the best approach to avoid further delay was to organize BMSB as an interim edition based on practical grounds.

At its meeting in Toronto in 1979, when the assignment of editors for the four volumes came under discussion, I must have looked like a deer caught in the headlights when Professor Murray looked at me over his glasses and proposed

that I should be the editor for Volume 1. Knowing what travail Norman Gibbons had gone through when he had edited the 8th edition, my mind was in turmoil about accepting such a task. I telephoned my Biology Department head, Robert A. Paterson, to ask if he thought I should do it, as it would undoubtedly interfere somewhat with my teaching and my research productivity. He was very supportive and urged me to accept. Consequently, editing Volume 1 became a major activity of mine for the next 5 years. My task was aided immensely by a perfect working relationship with Jack Holt, who was a wise counselor and who encouraged and supported me throughout.

In creating the format for the chapters, I thought that presenting most of the descriptive data in tables instead of essay form could save a lot of space. Toward that end, I devised a chapter format and some model chapters to serve as a guide for authors. Desk computers were not available at this time, and manuscripts were written on typewriters, with corrections being done with "whiteout" liquid (which I used in copious amounts). Some authors paid little attention to the format, not understanding that the *Manual* was a book and not merely a collection of essays. Although most authors faithfully followed the format, for others I sometimes had to separate the various portions of their manuscripts with a scissors and then paste them back together in proper order. Manuscripts and corrections were sent back and forth solely by "snail mail". The mailing costs from my department were huge, but Robert Paterson never complained. Although computer "spread sheets" were not available, my dad helped me create a special filing system whereby I could keep track of each manuscript at its different stages of preparation.

The editing was laborious, but I always kept in mind that the chapter authors were writing out of love of their science, not for money, and that is why *Bergey's Manual* is a truly noble publication. Except for the publishers, no one, including the editors, has ever received any remuneration for preparing the *Manual*. Bergey's Manual Trust is a nonprofit foundation and all royalties from sales of the *Manual* are used entirely to prepare the future editions.

As an editor, I had some contentious issues to deal with. In some instances, where multiple authors had been invited to write a chapter, they sometimes disagreed heatedly about the content, and I often had to act as an intermediary between the "warring factions". Irreconcilable taxonomic difficulties occurred with some taxa, such as a controversy over the classification of certain *Neisseria* species as *Moraxella* species, and these problems made it necessary to describe the same organisms in different chapters or include an editorial note explaining the problem to readers. One day, in the midst of a splitting headache, I received a telephone call from two authors informing me that they disagreed with the format and would not adhere to it, and unless they had their way they would pull out. After futilely trying to explain to them the difference between a book vs a collection of essays, and realizing that I would be unable to find another authority to invite to write the particular chapters, my head throbbed so much that I told them to do whatever they wanted. This is why there is one section in Volume 1 that differs from all of the others in its format. Other authors wanted to pull out unless they could use the term "serotype" instead of "serovar", despite the recommendation in the Code of Nomenclature. Several problems arose with a few authors who delayed submitting their chapters, thereby delaying publication of the entire *Manual*. One particular chapter had been promised for over 2 years; and with the deadline for sending the *Manual* to the printer only 1 month away, I repeatedly telephoned the author, only to be told he would not be able to write the chapter. Panic set in, but fortunately I found another expert on the same bacterial group who said he would write the chapter in 1 month. He did a first-rate job, and I will always be grateful to him for that kindness.

During the next few years, I spent a good deal of time updating the differential tables that had been in Volume 1 of BMSB for the forthcoming determinative version of the *Manual*, because new genera and species were continually being created and needed to be added. Other board members were doing similar updating, and in 1994, *Bergey's Manual of Determinative Bacteriology*, 9th edn, was finally published (Holt et al., 1994).

At this time, I was asked by Philipp Gerhardt to be the editor for the Systematics section in the *Manual of Methods for General Bacteriology* to be published by the American Society for Microbiology (Gerhardt et al., 1981). I thought this book would be very helpful to persons who needed to know how to characterize new taxa. Accordingly, I asked John Johnson to write the section on methods for genetic characterization (DNA–DNA and rRNA–DNA hybridization) and Rita Colwell and Brian Austin to prepare the section on numerical taxonomy methods. Robert Smibert and I undertook the task of writing the section on phenotypic characterization. This was a new style of writing for us, because the book was a "how-to-do-it" book and Phil Gerhardt rightly wanted the methods written in the imperative voice. I also wrote the chapter on Enrichment and Isolation, and assisted Phil on the chapter "Solid Culture". I found Phil Gerhardt to be an extremely competent editor and coordinator and it was a great pleasure to work with him. Because of the increasing use of molecular biology methods, the methods book was expanded and updated in 1994 with the title

renamed to *Methods for General and Molecular Bacteriology*. Robert Smibert and I continued to prepare the section of general characterization of bacteria, but I decided to drop the section on numerical taxonomy and add separate, expanded chapters by John Johnson on the similarity analyses of DNAs and rRNAs, as well as a new chapter by Joseph O. Falkinham III on nucleic acid probes.

In 1991, I "burned out" and resigned from the Board at the age of 62. With my courses and research program, and the increasing need to care for my parents who were now very old and frail, I felt that everything was closing in on me and that I just could no longer do a good job for Bergey's. I had always enjoyed working on the Manual and with the outstanding scientists I had come to know so well – Don Brenner, Marvin Bryant, Jack Holt, James Molder, Norbert Pfennig, Karl-Heinz Schleifer, Peter Sneath, Jim Staley, and Stan Williams. But Robert Murray had retired from the Trust, and that seemed like the end of an era to me. I thought that people much more knowledgeable than I would be better qualified to serve on the Board. However, I did miss the Trust's activities very much and I still attended some of the annual meetings.

In 1996, Stan Williams – then Chairman of the Trust – asked me informally if I wanted to return to the Board. I readily agreed, and the Board voted to reappoint me. I should note that this was the first time in Bergey history that a member who had once resigned was reappointed! I did retire permanently in 2002.

The health of my parents had grown progressively worse, and my mother was developing dementia. My dad begged me not to put him in a nursing home, so I hired caretakers who, in addition to me, saw to the needs of mother and dad at their home 24 hours a day. My dad died of multiple organ failure in 1997 at the age of 107, and my mother died of cancer a week later at the age of 95. They had been married for 72 years. Their deaths had a devastating effect on me for years.

In 1996, Jack Holt retired as Editor-in-Chief and was replaced by George Garrity. At that time there had been major changes at Bergey's publisher, the Williams & Wilkins Co., which had merged with another publisher. After re-examining our publishing arrangements, the Board decided to consider other publishing firms, and, mainly through the efforts of Stan Williams and George Garrity, we signed a new agreement with Springer-Verlag in 1998.

I was not involved with Volume 1 of the 2nd edition of BMSB, but I helped with the co-editing of Volume 2, which

Noel Krieg in 2008.

covered the *Proteobacteria* and was to be published in three parts. As I worked on this new task, I enjoyed an excellent working relationship with George Garrity, whom I greatly admired for his organizational abilities and his dedication to moving the *Manual* into the electronic age. It was quite a change for me to use a computer for communicating with authors and for receiving and editing their manuscripts. George was extraordinarily patient as I slowly learned the intricacies of electronic editing. Thanks to the 339 authors who gave selflessly of their time and effort, Volume 2 was published in 2005. I was minimally involved with Volume 3, which covered the Firmicutes and was well on the way by 2006.

I thought I was finished with *Bergey's Manual*, but Jim Staley – then the chairman of the Trust – somehow persuaded me into editing the particular sections on the phylum *Bacteroidetes*, the class "*Bacteroidia*", and the class *Flavobacteria* for the forthcoming Volume 4 of BMSB II. I knew little about these organisms, but I consulted the best authorities, such as Jean-François Bernardet and Sydney M. Finegold, for their advice. Acting on their recommendations, I invited authors for the genera in these groups and was once again editing manuscripts. Despite many problems with Volume 4,

I am glad to say that it has now been published. I am grateful for the splendid help given me by William B. (Barny) Whitman, the Director of the Editorial Office of Bergey's, and by Aidan Parte, the Managing Editor.

It was one of the great honors and privileges in my life to have served on the Bergey's Manual Trust and to have watched the dynamic growth and development of systematic bacteriology from this unique vantage point.

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Reflections on microbial systematics

Peter H.A. Sneath

I am honored to be asked to write an autobiographic account of my contributions to microbial systematics (Figure 1). But I have been sparing with bibliographic citations, because most of the relevant details can be found in the book with Robert Sokal (Sneath and Sokal, 1973) or in a review of numerical taxonomy (Sneath, 1995).

My father's family were farmers in south Lincolnshire, and my mother's were from a background in commerce and education in Leicestershire. They met because a girl in my father's village, Thurlby near Bourne, went into service as a housemaid in my mother's family home at Leicester, and my mother and father met on one of the visits to Thurlby.

My father was Alec Andrews Sneath, son of Henry Andrews Sneath and Elizabeth Sneath. H.A. Sneath was a straw and hay merchant in Thurlby. The family name Andrews commemorates my father's great-uncle, Henry Andrews, a Methodist missionary in Trinidad, who died there. My father read history at Manchester University and then entered the Methodist ministry, so my father's family were Methodists, as am I. He was a descendant of Henry Andrews, of Royston, who was a teacher and calculator to the Astronomer Royal.

My mother was Elizabeth Maud Adcock, daughter of Thomas Draper Adcock, a teacher, whose family were known for baking Melton Mowbray pork pies. He educated himself by reading while he worked up the pastry for the pies. My mother trained as a teacher at Homerton College.

My father went to Ghana in the Methodist mission, where my mother joined him at the start of World War I, and they were married there. My father served first at Cape Coast, Ghana, then in Sri Lanka, and after World War II again briefly in Ghana.

I was born in 1923 at Richmond College, Galle, Sri Lanka, where my father was Principal and my mother taught at the adjoining college for girls. So my early education was at Richmond. In 1932 the family came home on furlough for a year, and my brother Frank and I attended the village school in Thurlby. When my parents returned to Sri Lanka we both went to Wycliffe College in Gloucestershire. In 1936 we visited our parents, and soon after that my parents came back shortly before World War II. On the outbreak of war our school was evacuated to St David's College, Lampeter, in Wales, where we finished our secondary education.

Figure 1. Peter Sneath in 2007.

I was fortunate to get a scholarship to Cambridge University, and went up in 1941 to King's College. At this time I began to think of what career to follow. My college tutor persuaded me to read medicine, and I did three years of preclinical study. I became very interested in pathology, so my third year was in that subject. Because there was a great shortage of doctors for the armed forces I was not called up to the forces until after the war. So I did my clinical studies in 1945–1950 at King's College Hospital, south London. For part of this time the students were evacuated to Horton Hospital near Epsom, Surrey, which received many casualties from the bombing of London as well as from the armed forces. I then qualified in medicine and had three jobs as an intern, one in medicine, one in surgery and one in pathology, and then stayed to qualify as a Pathologist in the Royal Army Medical Corps. In 1950 I was posted to Malaysia and spent a year in Singapore and a year in Kuala Lumpur, looking after pathology and some medical wards.

On my return to Britain I enrolled at the London School of Hygiene and Tropical Medicine for the Diploma in Bacteri-

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ology. At the end of this academic year I married Joan Sylvia Thompson, the start of fifty-two happy years of married life. The Medical Research Council then employed me as a research worker at the National Institute of Medical Research at Mill Hill, north London, where I joined the Division of Microbial Physiology under Martin Pollock, FRS. It was then that my interests in microbial systematics began, and in 1964 I moved to the University of Leicester.

In the 1960s the University of Leicester was building up its science, and I was fortunate enough to be supported by the Medical Research Council as Director of the Microbial Systematics Unit. Our first laboratories were in a wing of what was originally a mental hospital after World War I. It still then had a padded cell! We did a good deal of teaching microbiology, and when the University started its Medical School in 1975 I was offered the chair in Clinical Microbiology, with part-time work for the Leicestershire Health Authority, until my retirement in 1989.

No one could have had a more devoted team. Dr Dorothy Jones was with me on the scientific staff; Michael Sackin was our computer officer; Michael Stevens (and later Margaret Bolton) was Chief Technician; with Hazel Lilley, and later Patricia Pell, as technicians. Dawn Starmer was secretary, followed by Brenda Jones and Sheila Hewitt. And over the years we had a number of research fellows and graduate students. I owe all of these, and many others, for their splendid support, and I am fortunate to have kept in touch with most of these (Figure 2).

I am also grateful to Martin Pollock, Sir Charles Harrington and Sir Harold Himsworth for encouraging the systematics of microbes, and to Sam Cowan, Director of the National Collection of Type Cultures, who gave me much support in the early days when the area was still obscure.

I cannot say just why I became a scientist. Both sides of my family had a background in teaching but none in science. From my school days I was fascinated by natural history. I had a small microscope, and built up a considerable collection of natural objects, many stained in splendid colors. I was fond of botany, zoology and chemistry, especially of zoology. Like many of my generation, I was greatly influenced by three books, Paul de Kruif's *The Microbe Hunters* and *Men Against Death*, and Hans Zinsser's *Rats, Lice, and History*.

My first interests in bacterial systematics centered on the purple pigment-producing bacterium *Chromobacterium violaceum*. I had isolated several strains from water in Malaysia, but unexpectedly found a strain from a fatal human infection. On returning to Britain I began to study these bacteria and another purple-pigmented species from temperate soils and water, *C. lividum* (now *Janthobacterium lividum*). This led me to reflect on how bacteria should be classified. All the current methods seemed inadequate: different methods used different criteria; this led to conflicting classifications. Slowly I began to realize that in bacteria, at least, there were no absolutely constant tests, so that strains must be grouped in a manner that took this into account – the degree of similarity between strains, based on a number of characters.

Figure 2. Peter Sneath with former student Christine Dodds at her inaugural lecture as Professor of Microbiology, University of Nottingham, 2008.

After much thought I concluded that bacterial species consisted of clusters of strains that shared many properties, though no character was necessarily constant. So a method was needed to identify these clusters, by comparing each strain with the others, and finding those strains that shared the most characters with one another. For a long time I was puzzled by uncertainty on how to weight different characters, until one day, on a London omnibus, I suddenly realized that all characters should have equal weight. Later it became clear that some characters had more information than others, so it should be that all units of information should have equal weight. This conclusion was reached after I had prepared my first papers on numerical taxonomy (Sneath 1957a, 1957b), and put in a footnote. I was grateful to virologist Alec Isaacs for telling me that my most important point was relegated to a footnote! Because equal-weighting had been proposed in a modified form by the French botanist, Michel Adanson, this approach is sometimes named Adansonian.

This analysis of strains was before the wide availability of computers. One government expert suggested that it only needed a Hollerith punched card sorter, though in the event the process became much more complicated than simple sorting. So the first study (Sneath, 1957b) used visual methods to estimate similarity, employing photographic images on X-ray film. The advent of computers gave new promise. My work was marvellously supported by one of the early British computer firms, Elliott Automatian, near Mill Hill. I well remember the first lecture on computer programming: the lecturer said "If you think you need not think any more, and that computers will do it all, let me tell you your thinking days are just beginning". Gerald Mills of Elliott Automation took me under his wing, and together we wrote the first computer program for Single Linkage clustering, on the Elliott 401 machine.

At that time computers were large machines, using electrical valves and without transistors, so they could fill a whole room. Input and output was by punched paper tape (the "ticker tape" of financial institutions). Instructions had to be in machine code, with one action for each cycle of the machine. For example, the 401 used a sonic loop (sound waves in a U-tube of mercury) for the immediate register, which took 32 machine cycles to multiply. Therefore one had to start the multiplication exactly 32 steps before one got the product. Dendrograms, tree-like diagrams, came from the work with Robert Sokal. In my first paper I represented the clusters by contour diagrams, which were soon superseded.

One day in 1958 virologist Christopher Andrewes, who was a keen entomologist, looked into my room and said there was another who was mad enough to classify organisms numerically. This was Robert Sokal writing in the *Proceedings of the Xth International Congress of Entomology* (Sokal, 1958) on classifying bees. A little later I was fortunate to receive a Rockefeller Fellowship to work on bacterial genetics with Joshua Lederberg. So in 1958–1959 I was in the USA, and when Lederberg moved from Madison, Wisconsin, to Stanford, California, we drove across the USA in what the family referred to as the Great Trek (one of my forbears had a sister who married a Mormon, and did indeed take part in the historic Great Trek to Utah.

There was an opportunity to visit Robert Sokal at the University of Kansas. We quickly hit it off, and started to think about the implications of numerical work in systematics. On my return to Britain I chanced to visit the Galton Laboratory in University College, London, and was surprised to meet Sokal again, who was on a sabbatical visit (Figure 3). We soon decided that we must write a book, and decided that the new discipline should be called Numerical Taxonomy

Figure 3. Peter Sneath and Robert Sokal, 1990.

(Sokal and Sneath, 1963).

There is a widespread impression that numerical taxonomy is entirely phenetic (based only on similarity), and never phylogenetic (interpreted as evolutionary ancestry). This is a misapprehension: Robert Sokal and I made it clear from the outset that it included phylogenetics to the extent that this was possible. At the time there were no molecular sequences available. It was right to sound a cautious note, in view of the obvious frequency of parallelism or back-mutations, and also the many problems of homology of non-molecular characters. Camin and Sokal (1965) in fact made one of the earliest attempts at reconstructing phylogenies.

It should be noted that these early questions, of homology and weighting, were (and still are in some respects) acute problems, and much had to be done to devise a consistent method for treating them. This was needed for both the phenetic and phylogenetic approaches. By and large they have withstood the test of time.

Today, with large numbers of molecular sequences available, very detailed phylogenies can be reconstructed, with good reliability. Instances of lateral gene transfer have turned out to be common, leading to complex relationships, but not so common as to disturb the main branches. However one should not mistake phylogenies for classification, though the two are deeply interwoven. Almost all current work, as Felsentein has pointed out (Felsentein, 2004) is phylogenetic, not classificatory. There are many areas where phenetic methods are useful: where there are no molecular sequences, or (particularly at the level of species and genera), the labor of finding them is too great; also when identification must depend on phenotypic properties. A few minor contributions were made here by me and my colleagues (Ambler et al., 1974; Sackin, 1971) but most of our work was phenotypic, including comparisons of electrophoresis traces (Feltham and Sneath, 1979).

Figure. 4. Richard Cowan, Peter Sneath and M.C. Vaughn at the Congress of Systematic and Evolutionary Biology, Boulder, CO, USA, in 1973.

Over the years I was able, with the help of the Medical Research Council and research grants, to initiate studies on a number of bacterial genera. Simple statistical principles showed that a large number of tests was needed for such work: thus one could not obtain a value of overall similarity of 90% from only two or three tests. So empirically we aimed for 50 tests or more, which could give accuracy of similarity to a few percentage. It became clear that rapid methods of testing were needed. At first we used Petri dishes divided into compartments (Sneath and Stevens, 1967), but as 96-well dishes and API testing kits became available we were able to adopt these successfully. Similarly, one needs a number of strains from a species if one is to characterize it reliably, and empirical tests imply that one should aim for at least 10 strains. A contribution to maintaining these numerous strains was made by Jones et al. (1984). Realization that many tests and strains are needed was thus perhaps the biggest advance in bacterial systematics for many years.

I am indebted to the people who fostered my interests in microbial systematics at the National Institute for Medical Research, Mill Hill, London. Martin Pollock was my boss in the Division of Bacterial Metabolism. He was interested in protein synthesis and worked extensively on penicillinase, to which I contributed a little. When I became interested in bacterial systematics he encouraged me to develop the subject, though it was not closely allied to his own interests in protein synthesis. Sir Charles Harrington was Director of the Institute, who read all the papers from it before they went for publication. He also encouraged me, and warmly approved of the production of the first book by Sokal and myself. Sir Harold Himsworth was Secretary of the Medical Research Council; he strongly supported the setting up of a Microbial Systematics unit at the University of Leicester.

A chance remark was made by an assessor for the Medical Research Council that no-one then knew the error rates of microbial tests. An informal group, the *Pseudomonas* Working Party of the Society for General Microbiology, undertook to study this. Two sources of uncertainty for systematics have been mentioned, that due to insufficient tests and that due to insufficient strains. But there are many other factors that determine reliability, in particular consistent test results.

The Working Party mainly worked on test reproducibility. The chosen bacterial strains were distributed, and the members did the tests in triplicate. Anonymity was ensured by referring to the different laboratories by numbers, whose key was known only to one assessor (and to this day I have no knowledge of it). The test methods were strictly standardized. One could then estimate the error between different laboratories, and that between the replicates from each laboratory. The results were published (Sneath and Collins, 1974) and they proved very illuminating. There was rather little difference between the laboratories. But some tests were conspicuously unreliable, much more than had been generally assumed. A very few were extremely consistent. So it was then possible to list the tests in order of reliability, and decide a cut-off level. This showed some tests should be excluded from such studies – those that showed more than about 10% disagreement. This has focussed attention on the field. It has to be remembered that biochemical tests do not suddenly become positive, because of their dependence of the length of incubation, so that some lack of reproducibility is inevitable (Figure 4).

A sequel to classification is identification, and in most of biology this is done by diagnostic keys, which are well-suited to organisms that have elaborate structure and where invariant characters of the species are usually readily available. But in bacteria such characters are few. Therefore alternative

strategies have been proposed. One of these is a polyclave, where a number of characters is compared with a reference set, and this number is increased until only one possible identity remains. This was originally based on overlapping punched cards, adding cards until only one punch hole remains through the stack of cards. This has not become popular, though it is quite a powerful method.

Instead the best method is the use of distance models. Distance is the complement of similarity. Thus similarity of 100% is zero distance. The bacterial strains can then be envisaged as points in a multidimensional space whose axes are defined by the characters. Each species is represented by a collection of strains (a cluster) in this space. The center of the cluster represents the most typical strain, and its radius determines the envelope within which the great majority of strains are formed. This data forms a reference library of the groups.

Distances in this model have a number of strange properties which may seem counter-intuitive, but they behave very much as distance in ordinary three-dimensional space. They also have statistical properties, because they can also represent probabilities. Thus one can imagine the various species as globes in space. An unknown strain is represented by a point in this space. It is possible to measure the distance of the unknown to the spheres, and find which it is nearest to. That will be the most likely identity. But in addition one can say whether the unknown is within the envelope of that sphere – which implies that it is extremely likely to be correctly identified. If the unknown is just outside the envelope the identification is less certain, and it may be an atypical strain. If it is midway between two spheres it may be a hybrid of the two species. And if it is a long way from any sphere it is a strain that cannot be identified from the existing data. Such strains, when further work is done, commonly turn out to be new species.

The first application of a distance system to bacteria was by Dybowski et al. (1963). This was greatly improved by Lapage and his colleagues at the National Type Culture Collection in London (Lapage et al., 1970); it was the first definitive study in this area. Our team at Leicester extended these concepts in various ways. They were published with geological examples in the journal *Computers & Geosciences* because its editor, my friend Daniel Merriam, wished to extend to geology as wide a range of computer methods as possible. These supplemented the basic system by giving methods for steps such as estimating the value of different characters, of finding the most diagnostic characters of a species, and of estimating the overlap of close pairs of species. All of these are useful in making and checking a new system. Illustrative examples are given by Williams et al. (1983a, 1983b) on *Streptomyces*, and by Jones et al. (1972) on *Streptococcus*.

It is remarkable how well these methods work when one considers the nature of the data, the lack of invariant characters, and the several sources of error and uncertainty. Algorithms for identification on these lines are now standard in automated laboratory instruments. And for those without computers the most common combinations of test results ("profiles") can be found and printed, so as to cover the great majority of likely identities (e.g., Clayton et al., 1986).

The success of numerical taxonomy on bacteria prompted the question whether it was applicable to viruses, a much more difficult area. The first study was a phenotypic analysis of common viruses (Andrewes and Sneath, 1958). This gave little new insight, but it made reasonable sense. It is now clear that reasonable classifications can be made, but at the time this was much less certain. But phenotypic properties of viruses suffer from the same drawbacks as those of bacteria, and no substantial advance was possible before molecular sequences of viruses could be obtained. Then, a brief study on influenza sequences was made, to see whether the evolution occurs in a steady manner or in erratic jumps, but this was difficult to say, both for lack of data, lack of knowledge on hybridization and other factors. Similarly it was not possible to know if evolution occurs in a zigzag manner, rather than following the shortest path, as is assumed in algorithms for phylogenetic reconstructions. Conceptual difficulties on the definition of zigzag evolution, however, prevented much insight here. Yet it remains a problem for all phylogenetic work, because if we do not know the answer, one cannot estimate the reliability of phylogenesis based on shortest path, rather than zigzag, assumptions.

Of more general interest was the observation that one of the plant geminiviruses, is a hybrid, by showing that the dendrograms based on two different types of sequence were clearly very different (Sneath, 1991).

One of the biggest problems in bacterial systematics was the existence of over 30,000 names in the literature. But only a few thousand could be equated with any well-founded species, because of their poor descriptions. This problem was addressed by the International Committee on Systematic Bacteriology, and its Judicial Commission. In the years before 1970, wide discussion was held, and a fuller account of these activities can be found in Sneath (2005).

It was decided to make a new starting date for names of bacteria, that is a date before which names were no longer recognized. In consequence the Bacteriological Code of No-

Figure 5. Bergey meeting in Ottawa, Canada, 1988. Left to right: Don Brenner, Peter Sneath, James Staley, Norbert Pfennig, James Moulder, Noel Krieg, John Holt, unknown, R. G. E. Murray.

menclature was completely revised. These were achieved mainly by two members of the Commission, Victor Skerman and Stephen Lapage.

The first requirement was the choice of relatively well-founded names, about 1700 in number, covering species, genera, and higher ranks. This did not prove too difficult, and specialists in various areas were most helpful with their advice. This idea was the brain-child of Victor Skerman. The outcome was the Approved Lists of Bacterial Names (Skerman et al., 1980); earlier names were no longer required for consideration, although provision was made for reviving old names under certain conditions. New names had to be registered in an official publication, which was then the *International Journal of Systematic Bacteriology*.

The second necessity was to completely rewrite the International Code of Nomenclature of Bacteria. This was undertaken by Stephen Lapage. The new Bacteriological Code (Lapage et al., 1975) had a clear vocabulary and logical order, and incorporated the changes required by the Approved Lists. In this I was only the steersman; others did the rowing. But the results turned out to be very satisfactory, and those responsible for the Codes of Botany, Zoology and Virology gave the bacteriologists the satisfaction of considering the new Bacteriological Code in their plans for revision of their Codes, particularly new starting dates, and the registration of new names.

It is remarkable how stable the analyses from 16S rRNA have been. The demonstration of the distinction between bacteria and archaea depended on this molecule studied by Woese and his colleagues, and also on 5S rRNA from the work of Hori and Osawa, whose independent observations have often been overlooked. 16S rRNA has given a compelling view of the relations between bacteria, but its utility at the species level is still not entirely clear. It is not certain whether all bacterial species can be defined and distinguished by it. And there is some evidence for genetic crossover of rRNA in closely related species (Sneath, 1993).

Molecular sequences will soon be routine for bacterial identification. Yet many species are now being proposed from a study of a single strain. This seems unsound, and it will still be necessary to describe new species from several strains, and to express their position and limits by their center and radius in multispace.

Molecular sequences will not solve all problems in systematics. There still remain some problems with phylogeny. The occurrence of lateral gene transfer is uncommon enough to raise no serious problems. There are problems with finding the correct root of a phylogenetic tree. But what phylogeny should be chosen if different molecules yield different phylogenies? This leads to the need for a new consideration of the purposes of classification.

It has become common to say that taxonomic groups should be phylogenetic groups, preferably based on the DNA of the entire genome. Yet consider the following case: the African ostrich and the South American rhea are flightless birds of very similar morphology, behavior and ecology. But the DNA work of Sibley and his colleagues clearly indicates that their genomes are more different than the genomes of many other birds, e.g., penguins and petrels. Do we therefore wish to consider ostriches and rheas as two very dissimilar phylogenetic groups, or as one tight phenotypic group? Other examples will doubtless arise, though I know of no recent review: possible cases are “living fossils”, like the New Zealand tuatara lizard and the coelacanth fish.

Evidently some parts of the genome evolve in different ways. In this example the genes that largely determine phenotypes have evolved slowly over the millions of years since the separation of ancestral ostriches and rheas by continental

drift. Other genes have evolved faster in a clock-like manner. Therefore which properties do we wish to summarize in the form of taxonomic groups?

I have always viewed taxonomy as a practical subject, a branch of information science. Our present methods have led to many sound conclusions. Yet there may be millions of species of microbes to be discovered. We should therefore reflect on whether our present Rules and methods are adequate.

It has been a great privilege to be associated with the Bergey's Manual Trust (Figure 5). It has been a constant background for much of my active years. This has been from the time of R.E. Buchanan, the wizard of names and steadfast advocate of the type system, to my friend Bob Murray, whose vision was the splendid *Systematic* volumes of Bergey. A last note is that the engagements of spouses, at the annual meetings, has always been a delight, for no members needed to prove themselves by competing with each other. A happy and successful venture into collegiate work in science.

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