

## Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms

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### A B S T R A C T

The application of molecular biological methods to study the diversity and ecology of microorganisms in natural environments has been practiced since the mid-1980s. Since that time many new insights into the composition of uncultivated microbial communities have been gained. Whole groups of organisms that are only known from molecular sequences are now believed to be quantitatively significant in many environments. Molecular methods have also allowed characterization of many long-recognized but poorly understood organisms. These organisms have eluded laboratory cultivation and, hence, have remained enigmatic. This review provides an outline of the main methods used in molecular microbial ecology, and their limitations. Some discoveries, made through the application of molecular biological methods, are highlighted, with reference to morphologically distinctive, uncultivated bacteria; an important biotechnological process (wastewater treatment); and symbiotic relationships between Bacteria, Archaea and Eukarya.

### Introduction

#### *Why Use Molecular Methods to Study Microbial Diversity and Ecology?*

Studies of microbial ecology, diversity, and evolution have always been intimately entwined. This was emphasized by the seminal work of Carl Woese and his coworkers, that showed that the main lineages of life were dominated by

microbial forms [e.g. 132, 134]. Comparative analysis of ribosomal RNA (rRNA) sequences indicated that all cellular life belonged to one of the three domains, Bacteria, Archaea and Eukarya [137]. The comparative sequence analyses further allowed the definition of the major lineages (phyla or divisions [132]) within the three primary domains. This not only provided the underlying phylogenetic framework that had been hitherto lacking in microbial ecology (and microbiology per se) [85, 133], but also ultimately allowed the development of tools to address a central dogma of microbial ecology: an inability to cultivate more than a small pro-

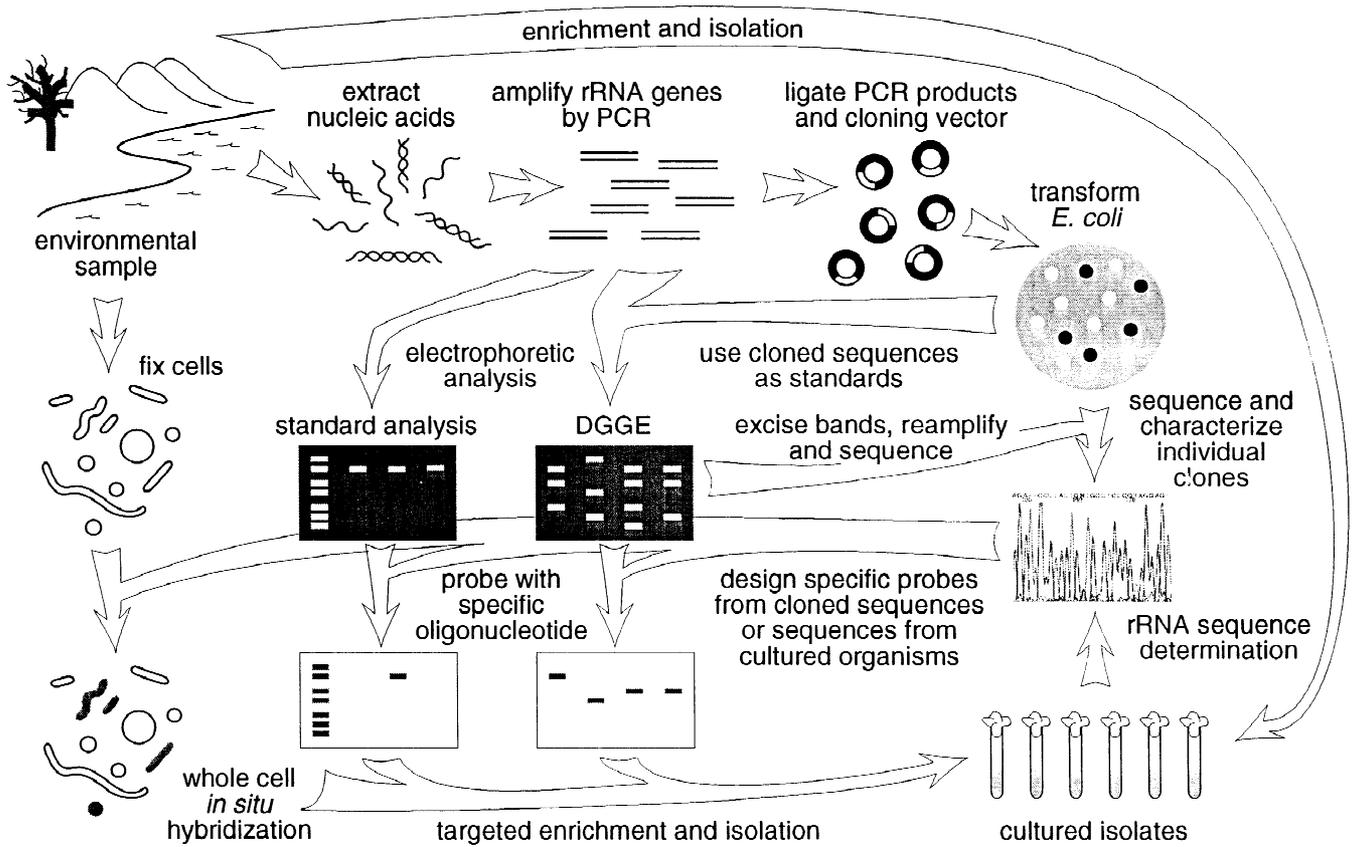


Fig. 1. Commonly used approaches in molecular microbial ecology.

portion (0.1–10%) of the bacteria that can be visualized by direct count procedures. This has been a considerable handicap to microbial ecology. Ecological inferences based on the metabolic properties of cultivated bacteria are, by necessity, unrepresentative of the natural populations from which they were obtained [15]. Although the biases of cultivation-based approaches were recognized by Winogradsky [131 cited in 127], it is only recently that means have been developed to study the uncultivated majority. First Zuckerkandl and Pauling [141], then Woese's advances in microbial phylogeny coupled with developments in molecular biology provided the necessary methods to allow the identity of uncultivated bacteria to be determined.

Norman Pace's group, in Indiana [84, 87], was among the first to appreciate the power of combining Woese's new phylogeny with molecular biology, and began what is now recognised as molecular microbial ecology.

#### *The Phylogenetic Basis of Molecular Microbial Ecology*

In principle, the techniques described below can be applied to any gene. The methods have been used, for example, to study aromatic hydrocarbon-degrading bacteria [98] and

mercury-resistant microbial populations [86]. Our discussion will, however, concentrate on the approaches involving analysis of ribosomal RNA sequences [5, 84, 87, 127]. An outline of many of the procedures commonly used in molecular microbial ecology are depicted in Fig. 1.

#### **The Nature of rRNA Molecules**

Due to the ubiquity of ribosomal RNA molecules (small subunit, 16S, and 18S, in Eukarya; large subunit 23S and 28S, in Eukarya) in all cellular life forms, comparative analysis of their sequences can be universally applied to infer relationships among organisms. The rRNA molecules comprise highly conserved sequence domains interspersed with more variable regions [48, 117]. In general, essential rRNA domains are conserved across all phylogenetic domains, thus "universal" tracts of sequences can be identified [45, 84]. In addition, it is also possible to identify sequence motifs of increasing phylogenetic resolution. For example "signature" sequences for the Archaea, Bacteria, and Eukarya have been recognized [45, 57, 103, 132], as well as short stretches of sequence characteristic of a number of the bacterial divisions and subdivisions ( $\alpha$ -,  $\beta$ -,  $\delta$ -,  $\gamma$ -Proteobacteria, high %G+C

Gram-positive bacteria, and the *Flavobacterium-Cytophaga* division [5, 74, 95]. Species- and subspecies-specific sequences have also been identified [5].

### Inferring Phylogenetic Relationships from rRNA Sequences

The most commonly used form of comparative rRNA sequence analysis involves the construction of phylogenetic trees. There are a number of procedures used to achieve this, but the first stage in these analyses is always the careful alignment of the rRNA sequences. This is a relatively straightforward task for regions that have highly conserved sequence. However, it is considerably more problematic in regions of greater sequence variability. Comparison with the secondary structure model of rRNA can often resolve these difficulties. The importance of careful alignment cannot be overstated. In any phylogenetic analysis, we must compare like with like if we are to be confident that a nucleotide substitution at any particular position in the sequence is, in fact, the result of an evolutionary event. Regions of sequence that cannot be unambiguously aligned are normally not included in phylogenetic analyses.

Once the rRNA sequences have been aligned, taking into consideration secondary structure interactions, phylogenetic analyses can be undertaken. Three widely used approaches to inferring phylogenetic trees are distance, parsimony, and maximum likelihood analyses [109]. The distance methods are conceptually the most simple. Pairwise comparisons of a set of aligned sequences are used to construct a distance matrix. The distances calculated are generally not simple binary similarities, but include a model of base substitution to account for multiple substitutions at a single site, for example, the Jukes and Cantor model [62]. The distance matrix can then be converted into a bifurcating phylogenetic tree by grouping the most closely related pairs of sequences. A useful overview of the principles of molecular sequence-based taxonomy is given by Schleifer and Ludwig [96]. Ribosomal RNA sequence analyses of this nature have been greatly facilitated by the availability of an excellent, indispensable, curated database of ribosomal RNA sequences (the ribosomal database project, RDP), based at the University of Illinois [72].

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## The Methods

### *The PCR-Clone-Sequence Approach*

The earliest attempts to analyze the diversity of naturally occurring microbial populations relied upon direct extrac-

tion, purification, and sequencing of 5S rRNA from environmental samples [see 84 and 87 for examples]. The limited length of the 5S rRNA molecule (approximately 120 nucleotides) means that there is limited scope for high-resolution phylogenetic analyses based on 5S rRNA sequences [84]. Consequently, this approach could be used only in the analysis of microbial communities with limited diversity [104, 105]. The development of robust and simple DNA cloning techniques and the polymerase chain reaction (PCR) have however, allowed higher resolution analyses of more complex communities using SSU rRNA sequence analysis [e.g. 46, 125]. The SSU rRNA molecule is approximately 13 times longer than the 5S rRNA, and thus contains considerably more information. The presence of universally conserved sequences at the 5' and 3' ends [30, 35] allows both the recovery of rRNA sequences as cDNA [129] and amplification of nearly complete SSU rRNA genes from DNA extracted from natural samples [14]. Only the latter procedure will be outlined, as this is currently the most widely adopted method of sequence retrieval from natural samples.

The starting point for this and related procedures is the extraction of nucleic acids of sufficient quality to permit activity of the enzymes used in subsequent procedures (Fig 1). This is not a trivial matter and will be discussed in relation to limitations of the methods.

The extracted DNA is subjected to PCR amplification using "universal" primers or primers designed to amplify rRNA genes from a particular group of organisms. The PCR product can then be cloned either by "filling" overhanging 3' deoxyadenosine residues and blunt-end ligation procedures, or by using commercially available kits for the cloning of PCR products. Alternatively, restriction sites can be incorporated in the amplification primer. Cloning of the PCR products can then be achieved by standard cloning method.

### Screening clone Libraries for rRNA Genes

Once cloned, the 16S rRNA gene library can be screened by a variety of methods. Colony hybridization procedures using rRNA gene-specific oligonucleotide probes of defined phylogenetic resolution may be used. However, the specificity of the probe used is important to avoid false positive signals at this stage.

Plasmid minipreps and restriction digests can be used to confirm the presence of cloned DNA of the correct size, or, alternatively, colony PCR (using, for example, sequencing primers with priming sites that flank the insert DNA) can be used as a rapid screening procedure to detect cloned PCR

products and can also rapidly provide template DNA suitable for sequencing.

### Sequencing of Specific Clones

Automated DNA sequencing systems have greatly facilitated the rapid screening and analysis of large gene libraries. Initial screening of rRNA gene-containing clones, by restriction fragments length polymorphism (RFLP) analysis of purified plasmid DNA or insert DNA obtained by colony PCR for the presence of near identical sequences, can greatly reduce the number of clones that require complete sequencing. Alternatively, single-lane sequencing can also be done to allow higher resolution screening [126].

Complete sequencing of the cloned rRNA genes is facilitated by the presence of conserved sequence domains throughout the molecule, allowing primers to be designed that permit sequencing of almost the complete rRNA gene [30]. Once a sequence database has been generated from the clone library, phylogenetic analyses can be carried out, and the diversity of the microbial population can be determined, with reference to previously published sequences [72].

### PCR and Diagnostic Oligonucleotide Sequences

The rapidly expanding database of rRNA sequences now contains several thousand sequences, and represents an invaluable resource. By comparison of the more variable regions of the molecule, it is possible to design oligonucleotides of varying phylogenetic resolution. These can be utilized in the detection and enumeration of specific groups of bacteria. Detection of specific organisms, without cultivation, can be achieved by PCR alone, or combined with the use of diagnostic oligonucleotide probes. Enumeration in PCR-based systems is problematic, and determining relative abundance [47] may be the best that can be confidently achieved within the limit of current technology. For absolute enumeration and spatial localization of specific microorganisms in natural samples, whole cell *in situ* hybridization techniques hold considerable promise [5, 102].

### PCR with Specific Primers

Specific primers have been used to amplify fragments of rRNA operons and other genes in order to detect the presence of specific organisms or groups of organisms in clinical specimens [130], foodstuffs [112], and environmental samples [58, 60]. The advantage of the method is that it can

be both highly specific and sensitive (detection of as few as three cells has been reported [115]). The main disadvantage is that it is difficult to make the system quantitative. Statistical methods based on most probable number estimations have been used [23], and the use of competitive internal standards have also been used successfully [29] in an attempt to obtain quantitative data from PCR-based analyses.

The sensitivity and specificity can be improved by adopting a "nested" approach to PCR, whereby initial amplification is carried out with a pair of primers with broad specificity. A second round of amplification is conducted on the product using primers with target sites internal to the first primer pair and of greater specificity. This has been successfully used to detect autotrophic ammonia-oxidizing bacteria in samples of lakewater, where the populations of these bacteria are generally around  $10^4$  cells liter<sup>-1</sup> [51]. Increased specificity and sensitivity can also be achieved by probing the initially amplified product with labeled (isotopically or otherwise) specific oligonucleotides.

### Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) [38] is a method by which fragments of DNA of the same length but different sequence can be resolved electrophoretically. This method has recently been applied to the analysis of 16S rRNA genes from environmental samples [83] and allows the separation of a heterogeneous mixture of PCR amplified genes on a polyacrylamide gel. Individual bands may be excised, reamplified and sequenced [39], or challenged with a range of oligonucleotide probes [83], to give an indication of the composition and diversity of the microbial community. DGGE is relatively rapid to perform, and many samples can be run simultaneously. The method is, therefore, particularly useful when examining time series and population dynamics. Once the identity of an organism associated with any particular band has been determined, fluctuations in individual components of a microbial population, due to environmental perturbations, can be rapidly assessed. This would be of particular use when studying microbial populations in large-scale biotechnological processes such as wastewater treatment, where the microbial population is treated, to a large extent, as a "black box," but where rapid changes in influent waste composition can have catastrophic effects on the microbiota, and hence the effluent quality. Inevitably, there are limitations associated with the technique. These include assigning particular bands to specific groups of organisms, particularly where multiple bands oc-

cur; where a band is assigned to a particular organism, fluctuations can be determined only, at most, semiquantitatively with this PCR-based assay.

### Whole-Cell Hybridization

Whole-cell in situ hybridization, with fluorescently-labeled oligonucleotide probes, for studies in microbial ecology was first developed in the late 1980s [24]. In recent years, the technique has been used successfully to analyze many ecosystems [5]. In short, the procedure involves fixing the sample (usually with paraformaldehyde or alcohol) to permeabilize the cells while maintaining their morphological integrity. The cells are either attached to gelatin-coated microscope slides or hybridized in suspension and immersed in hybridization solution containing a fluorescently labeled oligonucleotide. The sample is then incubated for 2–3 h to allow the probe to bind to complementary rRNA sequences. The optimal temperature for hybridization must be determined empirically to avoid binding of the probe to rRNA sequences with some mismatches with the probe. A more convenient method for optimizing probe hybridization is by the inclusion of different concentrations of formamide in the hybridization buffer [74], with hybridization conducted at a single temperature. Following hybridization, the sample is washed to remove unbound probe, and the sample is viewed by epifluorescence microscopy. Cells showing specific hybridization with the fluorochrome-labeled probe can be identified and enumerated. Counterstaining with DAPI (4',6-diamidino-2-phenylindole) [57] allows total cell counts to be determined.

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## Limitations of Molecular Microbial Ecology

While we have undoubtedly gained much new and valuable knowledge using the techniques described, as with all methods, there are important limitations that must be minimized, eradicated, or, at the very least, recognized. The limitations relate to the extraction of nucleic acids from natural samples, biases, and artifacts associated with enzymatic amplification of the nucleic acids, cloning of PCR products, and sensitivity and target site accessibility in whole-cell hybridization techniques.

### Nucleic Acid Extraction

A major limitation of all the methods described, with the exception of the whole-cell hybridization techniques, is the

quantitative recovery of nucleic acids from environmental samples. There is always the philosophical argument that if you do not know the total amount of nucleic acids present in a sample, then it is difficult to assess the efficiency of recovery by any extraction technique. This is compounded by the fact that spores will be less readily lysed than vegetative cells, and Gram-positive cells are more resistant to cell lysis than Gram-negative cells. While this is irrefutable, a reasonable indication of the efficiency of cell lysis in an environmental sample can be obtained by microscopic enumeration of the cells in a sample before and after lysis treatments. There are many published methods for extracting DNA from natural samples [16, 41, 59, 114], but there have been few systematic studies that have addressed this issue. It is possible that the same lysis technique may give different results with different types of sample such as water, sediment, or soil, and the degree of cell lysis should be determined independently. It has been demonstrated that a combination of physical and chemical treatments, such as freezing and thawing, lysis with detergents, and bead beating lysed approximately ninety-six percent of cells in soil and also lysed bacterial endospores with high efficiency [81]. It was noted however, that smaller cells (0.3–1.2  $\mu\text{m}$ ) were more resistant to lysis. This clearly has implications for recovery of sequences from environmental samples where many cells may be in a state of starvation and, hence are likely to be small. Other workers have found, however, that, even without harsh physical treatments such as bead beating, up to 99.8% lysis can be obtained [94], although this did require long incubations with lysozyme and up to six freeze-thaw cycles.

### PCR and Cloning

Selectivity in PCR amplification of rRNA genes is another source of bias that can affect the results of molecular biological measures of diversity. Small differences in the sequence of universally conserved regions may result in selective amplification of some sequences, particularly when primer annealing is at high stringency. The frequency of different sequence types in PCR-derived rRNA gene clone libraries has sometimes been assumed to represent the relative abundance of different components of a microbial community. This cannot be claimed with any confidence, as the copy number of rRNA genes present within the genomes of different organisms can range from 1 to 14 [21, 139]. Thus, assuming unbiased amplification, a mixture of equal cell numbers of *Bacillus subtilis* (10 rRNA operons) and “*Thermus thermophilus*” (2 rRNA operons) would produce a li-

brary that indicated a 5 to 1 greater number of *B. subtilis* in the original mixture. In this example, the copy number of the genes in each genome and the size of the genome of both bacteria are known, and this can be accounted for in our estimation of species abundance. In natural samples, we have no such information about the constituent microbial types. There is also concern that more abundant sequences are preferentially amplified, and low-abundance sequences are discriminated against [127]. It has been further suggested that high %G+C templates are discriminated against due to lower efficiency of strand separation during the denaturation step of the PCR reaction [93]. PCR amplification using artificial mixes of genomic DNA from organisms with different genome sizes and numbers of rRNA operons has demonstrated that, in general, the ratio of rRNA genes in the PCR product mix do, in fact, reflect the ratio in the starting mixture of DNA [37]. However, when rRNA operons were clustered on the genome, rather than evenly distributed, the clustered genes dominated the PCR products [37]. The implication of these results is that we can never confidently extrapolate from sequence composition in a clone library to a quantitative population composition in an environmental sample.

Suzuki and Giovannoni [108] have recently demonstrated that some primer pairs gave a strong correlation between the ratio of genes in the starting mix and the ratio in the final PCR product. Other primer pairs, however, produced mixtures of rRNA genes in the PCR product that tended toward a 1:1 ratio independent of the starting ratio of the genes. This effect was accentuated with increasing numbers of cycles. A kinetic model was devised that predicted the observed PCR bias and demonstrated that preferential reannealing of the template reduced the amplification efficiency with increasing numbers of PCR cycles (hence concentration of template). The tendency toward a 1:1 ratio was explained by the fact that if two templates were originally present, with one in excess, then the critical template concentration (at which amplification efficiency was reduced by (preferential template reannealing) would first be reached in the most abundant template. The less dominant template would continue to be amplified more efficiently until it reached a similar critical concentration. This occurred only with primer pairs that gave high amplification efficiency. Primers that gave lower yields of PCR product retained the initial ratios of different templates.

The authors ultimately suggested that, in environmental samples, this may not be a problem. It was argued that, where large numbers of different templates were present at

low concentrations, it was unlikely that any single template would be present in high enough abundance to result in preferential template reannealing becoming a problem. However, the presence of closely related sequences from different taxa was not considered, and it was cautioned that further work would be required to convincingly resolve this complex problem [108].

The work of Suzuki and Giovannoni is borne out by the observation that cloned PCR products generated using different primers resulted in significantly different composition of clone libraries [90]. Furthermore, this study found that the same batch of PCR product cloned using either blunt-end or sticky-end cloning procedures gave different results. However, it is not clear how internal restriction enzyme cleavage affected the results, since the clone libraries were screened by dot blot hybridization procedures and the size of the insert DNA in the screened clones was not reported. There has been no systematic attempt to assess cloning biases and their cause, but the inability to clone PCR products amplified from DNA extracted from some rhizosphere soil samples has been observed. A PCR product amplified from DNA extracted from a soil sample taken only a few millimeters further from a plant root could be readily cloned (A. G. O'Donnell, personal communication).

The fidelity of PCR amplification varies, depending on the particular thermostable DNA polymerase used (manufacturers have quoted misincorporation rates in the range of 0.000002%–1.3% for different thermostable DNA polymerases). Careful analysis of secondary interactions should, however, normally identify discrepancies due to misincorporation of nucleotides during PCR. Nevertheless, there is a danger that the presence of novel taxa may be assumed as a result of infidelity in DNA replication.

Furthermore, interoperon differences of up to 5% in rRNA gene sequence have been noted [78]. This degree of sequence divergence can be associated with rRNA sequences from different individual species, as well as intra-operon variability [19]. This is obviously of concern when making conclusions about biodiversity from data obtained with rRNA gene clone libraries. The problem is even more acute when considering interstrain variability of rRNA sequences, which have been reported to be up to 16% [19]. This degree of sequence divergence is associated with different, validly described species and even genera in some lineages.

The formation of chimeric PCR products has also been observed [70] in which fragments from two different sequences become fused during the amplification process. One study recently demonstrated that up to 30% of products

generated during coamplification of similar templates were chimeric [124]. The experimental conditions used may well have promoted chimera formation to some extent. Nonetheless, the results starkly demonstrated the considerable potential for chimera formation during PCR amplification.

A number of computer programs have been developed to help identify chimeric sequences [66], but these have difficulty in identifying chimeras when the two sequences from which the chimera is formed show greater than 85% homology. The programs may also indicate the presence of chimeric sequences even when none exist [66]. The programs are best used as a guide to the presence of chimeric sequences. The authenticity of a sequence should be confirmed by independent sequence analyses, using the putative chimeric fragments. Discrepancies in the secondary structures also aid in the identification of genuine chimeric molecules.

### *Whole Cell In Situ Hybridization*

While the complex problems of enumeration associated with quantitative analyses involving PCR do not hold for whole cell hybridization, a number of other methodological constraints do exist. These can be divided into four main categories: cell permeability problems, target site accessibility, target site specificity and sensitivity.

The first hurdle that must be overcome for in situ whole-cell hybridization to be successful is the entry of the probe into the cell. This is normally achieved by fixation with denaturants such as alcohols, or cross-linking reagents such as formaldehyde or paraformaldehyde. These fixation procedures not only aid in cell permeability, but also help maintain the cells' morphological integrity during hybridization. Simple fixation methods tend to permeabilize 70–90% of microscopically visible cells in aquatic samples [1], but, for some cells, additional treatment with solvents [50], acid [71], or enzymes [7] may be required.

Even when cell permeabilization has been achieved, there is no guarantee that probe hybridization to rRNA will occur within the cell. This is believed to be the result of the target sequence in the rRNA being inaccessible due to strong interactions with ribosomal proteins or highly stable secondary structure elements of the rRNA itself. This problem can normally be detected by a strong hybridization signal being obtained with a universal probe that is known to target an accessible site on the rRNA molecule. If another probe does not give a hybridization signal in the same cell(s), this generally indicates poor accessibility of the target site. An ex-

cellent review has been published recently that catalogues a list of successfully used target sites for rRNA-directed fluorescently labeled oligonucleotide probes in whole-cell hybridization [5]. The same review presents detailed discussion of the benefits and limitations of whole cell hybridization techniques.

Sensitivity of in situ hybridization is also an issue. In general, probes containing a single labeled molecule give a strong signal only if cells are metabolically active and, hence, contain large numbers of ribosomes and target rRNA (Fig. 2; 49, 75). A number of approaches have been taken to improve the sensitivity by using multiple singly labeled probes [2, 69], multiply labeled probes [113, 123], and enzyme linked probes or detection systems [4, 140] that allow signal amplification. In addition, the development of highly sensitive cameras has improved the sensitivity of in situ hybridization assays. A full discussion of methods for improving the sensitivity of in situ hybridization techniques is provided by Amann et al. [5].

As more rRNA sequences become available in sequence databases, the problem of probe specificity has become apparent, and design of diagnostic probes is becoming more difficult. While this problem has always existed, it is only with the rapidly expanding database of sequences that the problem has become more apparent. These problems are equally relevant to PCR and other oligonucleotide-dependent techniques, not only whole-cell hybridization. It has been pointed out that for an 18mer probe targeting a variable region of an rRNA molecule, there is a  $1:4^{18}$  chance of an unrelated target cell being detected. However, because even in variable regions there may be only a few positions that vary between taxa, the probability of detecting an unrelated cell is considerably increased ( $1:4^5$ , if only 5 positions are variable) [1]. It has been suggested that this problem can be overcome by using multiple specific oligonucleotide probes targeting different sites on the rRNA molecule and labeled with different fluorochromes [1]. An elegant solution based on this approach and taking advantage of additive color mixing with differently labeled probes has been demonstrated to work well and considerably reduce the detection of false positives [1].

Analogous to this approach is the use of specific PCR primers and confirmation of the identity of the amplified sequence(s) by the use of a specific oligonucleotide probe. While a single oligonucleotide target sequence may be found in a number of related taxa, the probability that target sites for three specifically designed oligonucleotides are found in a nontarget organism is much reduced.

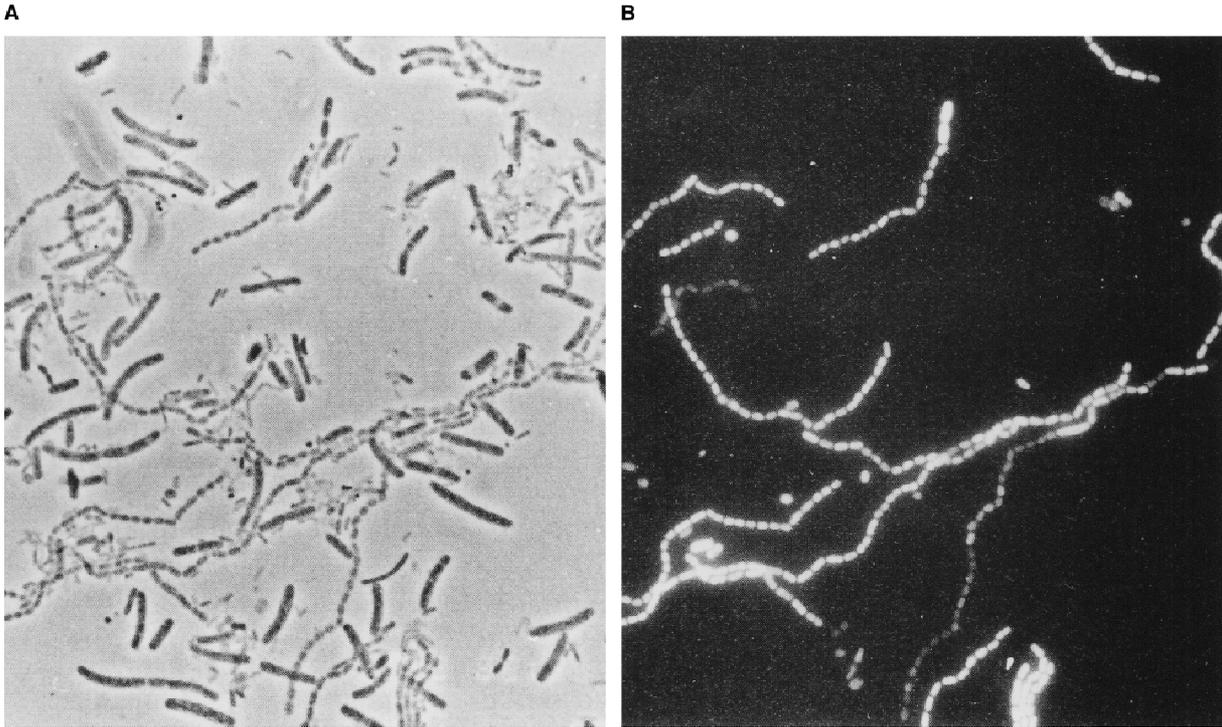


Fig. 2. Whole-cell in situ hybridization of sewage sludge. The images are of identical fields. **A**, Phase contrast image. **B**, Epifluorescence image. The cells were hybridized with a fluorescein-labeled oligonucleotide probe specific for *Acinetobacter calcoaceticus*. The probe specifically labeled filaments of cells identified morphologically as Eikelboom type 1863. This suggests that this morphologically identified group of organisms may be related to *Acinetobacter*. Bar = 10  $\mu$ m. Reproduced with permission [121].

### Some Key Discoveries Revealed by the Application of the rRNA Approach to Studies of Microbial Diversity and Ecology

Despite its limitations, this technology is permitting major advances in our understanding of microbial ecology and evolution. Its potential lies not only in the identification of specific organisms in the environment, but also in its ability to complement other methods (including classical microbiology and process-related studies), to assign them to functional roles, and to assess their significance in environmental processes. This section considers some examples of systems, previously refractory to classical techniques alone, where molecular analysis has played an expanding role.

#### *Morphologically Conspicuous but as Yet Uncultivated Bacteria*

##### Magnetotactic Bacteria

Magnetotactic bacteria were first described over two decades ago [11] and were perhaps considered microbial oddities. Some 20 years subsequent to their original description, we

now know that they are common in many freshwater and marine sediments [73]. In some environments, a single species of magnetotactic bacterium has been shown to be the dominant component of the bacterial population (e.g., the microoxic zone of a German freshwater lake [101]). Magnetotactic bacteria are characterized by the presence of intracellular, single-domain magnetic inclusions, termed magnetosomes, that serve to orient the bacteria along the earth's magnetic field lines. This may have a role in maintaining the bacteria in the microoxic zone of sediments, where many magnetotactic bacteria are found.

The difficulty in cultivating magnetotactic bacteria has hampered progress in the understanding of these interesting organisms. However, magnetic enrichment procedures [100] have been used to obtain relatively purified preparations of magnetotactic bacteria from freshwater sediments. These were used to obtain 16S rRNA gene sequences from which fluorescently-labeled oligonucleotide probes were designed and used to determine what cells were the source of the sequences obtained. This revealed the presence of three morphologically similar but phylogenetically distinct magnetic cocci. In addition, the different magnetic cocci identified

using whole-cell hybridization had characteristic tactic behavior [100]. All of the magnetotactic cocci belonged to the  $\alpha$ -Proteobacteria, though forming a lineage distinct from the cultivated magnetotactic spirilla that were also members of the  $\alpha$ -Proteobacteria. The first report of a cultivated magnetic coccus was published in 1993 [25], and it proved to be related to the uncultivated species reported by Spring et al. [100]. The same study reported the phylogenetic position of two cultivated magnetotactic vibrios that had identical 16S rRNA sequences and formed another novel lineage within the  $\alpha$ -Proteobacteria. The 16S rRNA sequence of a multicellular magnetotactic organism that could not be cultivated from magnetic enrichments was found to be related to sulfate-reducing bacteria in the  $\delta$ -Proteobacteria. Interestingly, unlike the  $\alpha$ -Proteobacterial magnetotactic bacteria that contain magnetosomes formed from magnetite ( $\text{Fe}_3\text{O}_4$ ), the  $\delta$ -Proteobacterial species contained greigite ( $\text{Fe}_3\text{S}_4$ ) magnetosomes, and the authors suggested that greigite-based magnetotaxis evolved separately from magnetite-based magnetotaxis. However, an uncultured, magnetite-containing bacterium from a freshwater lake in Germany was found to occupy a novel lineage within the bacteria [101], indicating that there may have been multiple origins of magnetite-based magnetotaxis.

### *Epulopiscium fishelsoni*

The gut of a number of species of surgeonfish (family Acanthuridae) are known to harbor large (up to  $80 \times 600$  mm) symbiotic microorganisms. These were originally described as eukaryotic protists because of their large size [40]. The ultrastructure of the bacterium, as determined by electron microscopy, was, however, more characteristic of prokaryotic organisms [20]. Like many of the magnetotactic bacteria, *E. fishelsoni* remains uncultured, so taxonomic inferences based on the biochemistry or physiology of the organism have been difficult to establish. Angert et al. [6] used the PCR to obtain 16S rRNA sequences from *E. fishelsoni* cells purified by micromanipulation. Comparative sequence analyses demonstrated that three sequence types obtained from *E. fishelsoni* purified from Australian surgeonfish formed a monophyletic group related to *Clostridium* spp. in the low mol% G+C Gram-positive bacteria. Furthermore, oligonucleotide probes designed to target the 16S rRNA from *E. fishelsoni*-like symbionts of an Australian surgeonfish also hybridized with symbionts present in the gut of surgeonfish indigenous to the Red Sea. This suggests that geographically isolated populations of these symbionts were related.

### Microbial Ecology of Morphologically Distinct Bacteria Important in Wastewater Treatment

A combination of labeling with fluorescent oligonucleotides and DAPI staining has proved particularly useful in the analysis of biofilms and sludges associated with biotreatment processes [e.g. 74, 119, 120, 121] and has already uncovered inconsistencies in our understanding of these processes. Traditional thinking, based on viable counts of bacteria, suggests that the bulk of bacterial biomass in activated sludge is moribund. However probing with fluorescently-labeled oligonucleotides suggested that up to 90% of the biomass present was metabolically active [119]. Furthermore, evidence obtained using culture-based techniques has implied that removal of phosphate from wastewaters was associated with bacteria of the genus *Acinetobacter*. Enumeration of these bacteria by whole-cell hybridization techniques, however, indicated that they constituted only a small proportion of the bacterial population in enhanced biological phosphate removal plants. This was contrary to results obtained from parallel culture-based studies [121].

Activated sewage sludge contains a considerable diversity of microorganisms, both prokaryotic and eukaryotic. Filamentous bacteria in sewage sludge have long been defined morphologically with little or no information on the physiology or evolutionary relationships of the bacteria observed [31, 32]. A number of these filamentous forms have been associated with operational problems in sewage treatment plants, such as sludge bulking (e.g., *Thiothrix* spp. and *Beggiatoa* spp.) and foaming (e.g., "*Microthrix parvicella*") that cause problems with solids separation. However, a number of these filamentous species are difficult to distinguish, even morphologically, and some are known to exhibit nonfilamentous growth. Wagner et al. [120] developed a range of oligonucleotide probes targeting filamentous bacteria characteristic of activated sludge. A suite of probes that could distinguish *Haliscomenobacter hydrossis*, *Thiothrix nivea*, *Leucothrix mucor*. Eikelbloom type 021N, some strains of "*Leptothrix discophora*," and *Sphaerotilus natans* were used to analyze the bacterial communities of ten German activated sludge plants.

The whole-cell hybridization approach successfully identified different filamentous bacteria in the activated sludge samples analyzed. It offered advantages over conventional morphological identification in that filaments deep in sludge flocs could be readily visualized, and large cocoid cells thought to be gonidia of *Thiothrix nivea* were identified, as were two morphologically similar, but distinct, filament

types. These would not have been distinguished by conventional methods of examination.

Two of the plants examined in this study were suffering from sludge bulking at the time of sampling. In one of these, the only filamentous type present was *Thiothrix nivea*, as determined by whole-cell hybridisation with fluorescently-labeled diagnostic oligonucleotides. *T. nivea* was, thus, confirmed as being responsible for sludge bulking in this particular plant [120]. Although *Thiothrix nivea* was detected in a number of plants not experiencing bulking, no quantitative data on the abundance of the filaments was presented. Therefore, the magnitude of specific populations could not be related to solids separation problems.

"*Microthrix parvicella*" is a filamentous Gram-positive bacterium associated with activated sludge bulking and foaming. On the basis of morphological descriptions, this bacterium was the most prevalent filamentous bacterium associated with instances of sludge bulking in Europe [32]. "*M. parvicella*" is notoriously difficult to maintain in pure culture [10], and little information has been obtained regarding the physiology and taxonomy of the bacterium. Recently, however, pure cultures of "*M. parvicella*" have been obtained by painstaking micromanipulation of individual filaments onto different growth media. After 5 weeks of incubation, colonies were just visible on agar plates. Small amounts of biomass scraped from agar plates provided sufficient DNA to allow 16S rRNA genes to be amplified and sequenced. Phylogenetic analyses placed "*M. parvicella*" with actinomycetes in the high mol% G+C Gram-positive bacteria [9]. This was particularly interesting since, previously, filamentous bacteria from the high mol% G+C Gram-positives had been identified in activated sludge using broad specificity 23S rRNA-targeted probes [95].

With the availability of rRNA sequence data and specific probes for these taxa, it will now be possible to objectively examine the phenomena of sludge bulking and foaming and to determine the changes in population structure and morphology associated with the problem. Physicochemical measurements of treatment processes combined with relatively rapid assessment of the microbial communities present will help overcome the "black box" philosophy that has dominated our understanding of biological wastewater treatment systems.

#### *Achromatium oxaliferum*

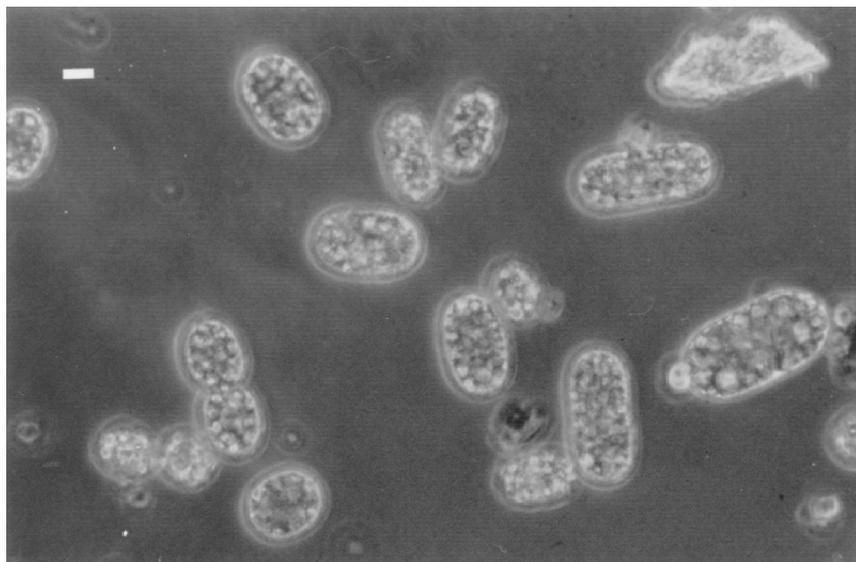
Besides chapters in reference texts such as *The Prokaryotes* [68] and *Bergey's Manual* [67], there have been few recent publications on *A. oxaliferum*. This is, perhaps, surprising

for a bacterium that has been known for over a century, particularly when *A. oxaliferum* is such a striking organism (Fig. 3).

*A. oxaliferum* is a remarkable bacterium. It can be greater than 100  $\mu\text{m}$  in length and up to 30  $\mu\text{m}$  in diameter. It deposits intracellular sulfur and is unique among the Bacteria in precipitating intracellular calcite inclusions [22, 54]. It is believed to be a sulfide-oxidizing autotroph [68], but no direct evidence for this has been reported. Until recently, the taxonomic position of *A. oxaliferum* was as a genus *incertae sedis* of the nonfruiting, nonphotosynthetic gliding bacteria [67].

The paucity of information about *A. oxaliferum* is due, in large part, to the continuing inability to cultivate the bacterium. Fortunately, cells of *A. oxaliferum* can be readily purified from crudely screened sediments by virtue of their high specific gravity conferred by the intracellular calcite inclusions [22, 55]. This has allowed sufficient purified DNA to be extracted to allow PCR amplification and sequencing of 16S rRNA genes [55]. Direct sequencing of PCR products amplified from *A. oxaliferum* DNA gave poor sequence data, but cloning of the PCR product and sequencing a number of the clones revealed substantial heterogeneity in the sequences obtained from purified cells. Three major lineages were apparent (Fig. 4) from the eight clones partially sequenced. The cloned sequences, however, formed a strongly supported monophyletic group within the  $\gamma$ -Proteobacteria. The subdivision of the  $\gamma$ -Proteobacteria within which the *A. oxaliferum* sequences fall is dominated by sulfide-oxidizing autotrophs. These include both symbionts of marine invertebrates and free-living, sulfide-oxidizing autotrophs and mixotrophs, including *Thiothrix* spp., *Beggiatoa alba*, and *Thioploca* spp. (Fig. 4). In addition to the sequence diversity observed in homogeneous population of morphologically conspicuous bacteria, electron microscopy (EM), employing a novel freeze-spray fixation procedure [55] that shrinks back the cell envelope to reveal details of the internal inclusions, demonstrated that distinct morphological types were also present. The morphological types that were characterized by the size and number of calcite inclusions, could not be distinguished by light microscopy. *A. oxaliferum* cells were confirmed as the source of the most dominant sequence type in the clone library by whole-cell hybridization techniques. However, it was impossible to associate specific EM-defined morphotypes with specific sequence types by using whole-cell hybridization techniques with sequence-specific oligonucleotides. Careful EM and molecular biological analyses of intact sediment samples will be required to

A



B

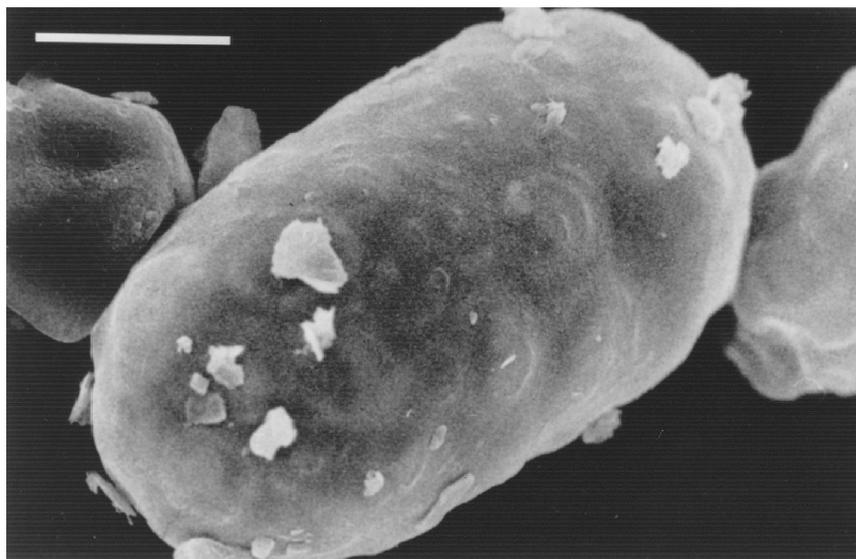


Fig. 3. **A**, Light micrograph of *A. oxaliferum* cells showing intracellular calcite inclusions. **B**, Electron micrograph of *A. oxaliferum*. The cells were fixed by freeze spraying. This results in shrinkage of the cell envelope revealing intracellular inclusions below the cell surface. Scale bar = 10  $\mu$ m. Reproduced with permission [54].

confirm that different morphotypes are associated with different sequence types, and not simply a result of the physiological condition of the cells.

### Symbiotic Relationships

#### Anaerobic Ciliates

Many protists are known to harbor endosymbiotic Bacteria and Archaea. For example *Paramecium caudatum* is infected by a number of bacterial symbionts of the genus *Holospora*. Comparative sequence analysis demonstrated that these bacteria were related to other intracellular parasites, the rickettsias, within the  $\alpha$ -Proteobacteria [3].

Most anaerobic ciliates lack mitochondria and rely upon fermentative metabolism and substrate level phosphorylation to provide energy for the cells. Most of the anaerobic ciliates, however, contain hydrogenosomes [82] that produce ATP, carbon dioxide, and hydrogen. Elevated partial pressures of hydrogen within the symbiont cells are inhibitory, and some mechanism for its removal must exist. To overcome this problem, many of the anaerobic ciliates have evolved symbiotic relationships with syntrophic endosymbiotic Archaea. It was suggested that the endosymbionts were hydrogen-oxidizing methanogens because of their autofluorescence, indicative of the presence of cytochrome  $F_{420}$ , a methanogen-specific electron carrier. Furthermore, the an-

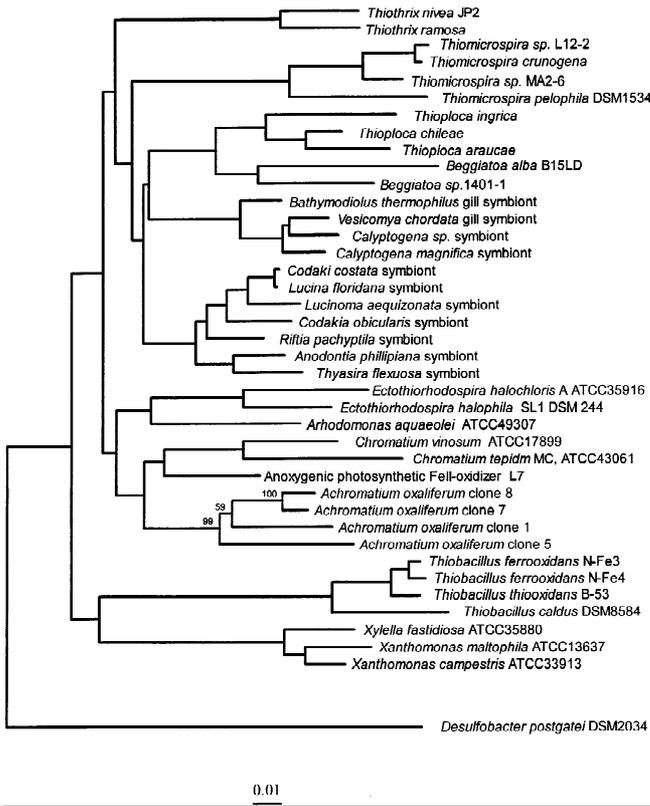


Fig. 4. Phylogenetic relationship between *A. oxaliferum*-derived 16S rRNA sequences and those from other morphologically distinctive sulfur bacteria.

aerobic ciliates produce methane; addition of bromoethane sulfonic acid (BES), an inhibitor of methanogenesis, results in accumulation of hydrogen. Electron microscopy studies revealed that the cells of the symbiotic Archaea are intimately associated with the hydrogenosomes [33, 34]. Recently, molecular methods have contributed to understanding the nature of the methanogenic symbionts, the specificity of the relationships, and how they evolved.

These methods have revealed that the Archaeal endosymbionts from a number of anaerobic ciliates are phylogenetically related to, but distinct from, known free-living methanogens. Archaeal endosymbionts from the Methanomicrobiales and the Methanobacteriales were identified, but none from the other main group of methanogenic Archaea (Methanococcales). A very small number of ciliate taxa have been investigated to date, and further investigations may reveal that methanogenic symbionts are even more diverse. As in many studies of this kind, the identity of the symbionts determined using molecular methods was different from that determined using culture-based methods [33, 34], and the use of in situ hybridization techniques confirmed that

the rRNA gene sequences recovered by the PCR had genuinely come from the symbionts.

Interestingly, identical isolates of putative methanogenic endosymbionts were obtained from phylogenetically distant protists *Metopus striatus* (a ciliate) and *Pelomyxa palustris* (an amoeba) [33, 34]. Also, different strains of *Metopus contortus* harbored distinct endosymbiotic Archaea, indicating that the host-symbiont relationship was not highly specific.

Parallel phylogenetic studies of the host protists demonstrated that there was no evidence of parallel evolution of host and symbionts. Thus, it was suggested that the protist-methanogen symbiosis probably evolved independently on several occasions [33, 34].

The associations within anaerobic ciliates have been taken a step further, by examining the phylogeny of the hydrogenosome-containing anaerobic ciliates [36]. Sophisticated phylogenetic analyses further demonstrated that different ciliate lineages had evolved hydrogenosomes on at least four independent occasions. The likelihood of the complex changes required for development of a hydrogenosome from an endosymbiotic bacterium occurring on four occasions was considered to be low. The authors suggested that an ancestral mitochondrion may have acquired the hydrogenase enzyme and other enzymes characteristic of the hydrogenosome, but not found in mitochondria, on four separate occasions. Another hypothesis would be that the original endosymbiont had already acquired a hydrogenase and other hydrogenosome associated enzymes (e.g., pyruvate-ferredoxin oxidoreductase) before evolving into the hydrogenosome. These hypotheses may now be tested. Without the use of molecular techniques, it is likely that questions on the origin and evolution of the endosymbionts and hydrogenosomes could not be addressed.

### Symbionts of Marine Animals

A diverse range of animals are known to harbor symbiotic bacteria. A number of particularly interesting examples are found in marine vertebrates and invertebrates. The symbiosis can range from facultative symbiosis, as is found with some bacteria inhabiting the light organs of flashlight fish, to obligate symbiosis, as is found among chemoautotrophic, sulfur-oxidizing symbionts and their gutless invertebrate hosts.

*Symbiotic Bacteria in Light-Organs of Marine Fish.* Symbiotic, light organ bacteria are known to occur in 22 families of fish [52]. In most of these, the symbiotic bacteria are readily cultivated and belong to three species of the genus

*Photobacterium* [56]. They are apparently indistinguishable from free-living members of the genus *Photobacterium* found widely in marine environments. It is believed that these facultative symbionts colonize the fish light organs, from the gut (which is connected with the light-organ in these fish). In support of the hypothesis that colonization by particular *Photobacterium* species from the environment occurs, it has been found that the light organs from deep-sea fish contain *Photobacterium phosphoreum*, characteristic of deep waters; whereas fish dwelling in shallow waters contain *Photobacterium fischeri*, indigenous to shallow water. In the flashlight fish of the family Anomalopidae and the deep-sea anglerfish of the suborder Ceratioidei, it has proved impossible to cultivate the bacteria that inhabit the light organ and the bioluminescent lure, respectively, of these two groups of fish. Thus, the nature of the symbionts and their source are unknown. The polymerase chain reaction was used to amplify 16S rRNA genes from the bacterial symbionts from the light organs of several genera of flashlight fish and deep sea angler fish, in an attempt to characterize them. The light organs proved to contain bacteria that shared a common ancestry with the facultative, symbiotic, luminescent bacteria of the genus *Photobacterium* and nonsymbiotic luminescent *Vibrio* spp. [52]. The uncultured symbionts however, constituted two new lineages within this radiation of the  $\gamma$ -Proteobacteria. Interestingly, the symbionts from different flashlight fish constituted a novel monophyletic group that was distinct from a second, monophyletic group formed by the symbionts from the anglerfish. There is, therefore, a possibility that coevolution of the symbionts and the host fish has occurred.

*Symbionts of Marine Invertebrates.* A number of marine invertebrates are known to harbor symbiotic bacteria. Interesting symbioses exist between the sulfur-oxidizing, chemotrophic symbionts of vestimentiferan worms and bivalves from submarine hydrothermal sites and sulfidic muds. Methanotrophic symbionts are found in some mussels.

Sulfur-oxidizing endosymbionts were among the first uncultivated bacteria to be analyzed using ribosomal RNA-based techniques. Total RNA was extracted from a homogenized symbiont containing tissue from the gutless tube worm *Riftia pachyptila*, the giant clam *Calyptogena magnifica*, and *Solemya velum*, a mussel that inhabits sulfidic tidal mudflats. Bacterial 5S rRNAs were purified from the homogenized tissues and sequenced [104]. Phylogenetic comparison with 5S rRNA sequences from a number of

free-living, sulfur-oxidizing bacteria placed all of the bacteria in the  $\gamma$ -Proteobacteria [106]. Subsequent analysis of 16S rRNA sequences from the symbiont bacteria, purified by density gradient centrifugation techniques, confirmed this phylogenetic placement, and also that the relationship between host and symbiont were rather specific [27, 28]. The sequence data suggested that symbionts from related invertebrates such of symbionts from within the family Vesicomidae formed monophyletic groups, whereas symbionts from more distantly related hosts including symbionts from members of the Vesicomidae (*Vesicomya* spp., *Calyptogena* spp) and the superfamily Lucinacea (*Codakia* spp., *Lucina* spp., *Lucinoma* spp.), were phylogenetically distinct (Fig. 4. [28]). One explanation for this observation is that the host-symbiont relationship based on chemolithotrophic sulfur-oxidation evolved independently on several occasions.

A combination of 16S rRNA sequence analysis and PCR analysis has also been used to demonstrate that putative symbiotic sulfur-oxidizing bacteria, cultivated from the bivalve *Thyasira flexuosa* [138], were, in fact, most likely surface contaminants of the gill tissue from which the bacteria were isolated; the true symbionts were not closely related to the cultured isolate [26].

The use of in situ hybridization techniques have also helped to unravel the mystery of symbiont transmission in these seemingly obligate associations. In the case of the vestimentiferan worm *Riftia*, this apparently occurs by reinfection of the larvae by free-living bacteria, since the symbionts are not associated with the sexual organs of the worm, and the larvae do not contain the symbiotic bacteria [18, 27, 44]. In the case of vesicomid clams of the genus *Calyptogena*, it was discovered that symbiont 16S rRNA sequences amplified from the ovaries of the clam were identical to the symbiont sequences recovered from somatic tissues. Furthermore, in situ hybridization with oligonucleotide probes confirmed that the symbionts were specifically associated with ovarian tissues. This suggested that vertical transmission was the means by which the symbionts were maintained [17]. This could not have been discovered without molecular biological studies since elucidation of such transmission mechanisms had previously only been possible when both the host and symbiont could be cultured in the laboratory. In the case of the hydrothermal vent invertebrates and their bacterial partners, neither could be cultivated in the laboratory.

#### *Ammonia-Oxidizing Bacteria*

Autotrophic ammonia-oxidizing bacteria are an ecologically important and physiologically specialized group. They are

responsible for the oxidation of ammonia to nitrite, the reaction that drives the process of nitrification in a wide range of environments [51]. The organisms are obligate chemoautotrophs. They all exhibit an essentially identical central metabolism. Carbon dioxide is fixed via the Calvin cycle, and ammonia is oxidized to nitrite to produce energy and reducing power. A paucity of diagnostic phenotypic characters in this group of Bacteria means that the described genera are distinguished solely on morphological criteria [13, 64]. All other phenotypic characters investigated such as cellular fatty acids, physiological characteristics, and cytochromes are essentially identical among the different genera [12, 43, 28]. Within the morphologically-defined genera species, designations have been defined largely on the basis of genotypic data including mol% G+C and DNA-DNA hybridization [63, 65]. The lack of useful phenotypic characters in the autotrophic ammonia-oxidizing bacteria makes them ideal candidates for the application of rRNA-based taxonomy.

Furthermore, the study of autotrophic ammonia-oxidizing bacteria in natural environments using culture-based methods is problematic. Molecular biological methods provide a useful alternative approach. Autotrophic ammonia-oxidizers are notoriously difficult to isolate in pure culture from environmental samples. Repeated sequential enrichment in ammonium salts medium is the method most frequently employed; it results in a collection of isolates that may not be representative of the diversity that exists in the environment [8]. Laboratory enrichment would be expected to favor those species that exhibit the fastest growth, irrespective of their activity under natural environmental conditions. For example, studies on the physiology of ammonia-oxidizing bacteria have been almost exclusively centered on *Nitrosomonas europaea* [89], because of the comparative ease with which this species can be grown in culture. This has resulted in ammonia oxidation in the environment often being equated with the activity of nitrosomonads [88, 99]. This sort of generality can lead to gross misconceptions in microbial ecology. There are now a number of reports in which the application of molecular biological techniques has revealed microbial populations whose compositions are not represented by those isolates found in culture collections [e.g. 42, 46].

#### Phylogenetic Diversity of Cultured Autotrophic Ammonia-Oxidizers

Data from 16S rRNA catalogues [135, 136] first demonstrated that there are two phylogenetically distinct groups of autotrophic ammonia-oxidizing bacteria. One of these con-

tained *Nitrosococcus oceanus* and was within the  $\gamma$ -subdivision of the Proteobacteria. The other contained *Nitrosococcus mobilis* and representatives of all of the other described genera of ammonia-oxidizers and was located within the  $\beta$ -subdivision of the Proteobacteria. The ammonia-oxidizing bacteria in the  $\beta$ -subdivision formed three deep branches represented by, respectively, *Nitrosococcus mobilis*, *Nitrosomonas europaea*, and a branch containing *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* strains. Each genus was represented by a single strain, and any taxonomic conclusions were further constrained by the cataloguing method, utilizing only 40% of the total sequence. Further investigation focused on partial sequences of the 16S ribosomal RNA genes of eleven autotrophic ammonia-oxidizing bacteria [53]. This confirmed the conclusions drawn from cataloguing data. The autotrophic ammonia oxidizers were recovered in two major lines of descent within the Proteobacteria: *Nitrosomonas* spp., *Nitrosococcus mobilis*, and strains of *Nitrosovibrio*, *Nitrosospira*, and *Nitrosolobus* in the  $\beta$ -subdivision (Fig. 5), and *Nitrosococcus oceanus* strains in the  $\gamma$ -subdivision. The autotrophic ammonia-oxidizing bacteria of the  $\beta$ -Proteobacteria formed a coherent group that could be interpreted as representing a single family. Within this clade, the genera *Nitrosovibrio*, *Nitrosospira*, and *Nitrosolobus* exhibited very high levels of homology in their 16S ribosomal RNA gene sequences; they can be accommodated within a single genus. Separation of these genera is currently based entirely on gross morphological differences, and these can now be considered more appropriate for the identification of species within this group. It was therefore proposed that the  $\beta$ -subdivision ammonia-oxidizers could be accommodated in two genera; *Nitrosomonas* and *Nitrosospira* which includes *Nitrosolobus*, *Nitrosovibrio*, and *Nitrosospira* strains [53].

This interpretation has been challenged recently [110]. It was suggested that, while *Nitrosospira* and *Nitrosovibrio* were sufficiently similar to warrant their inclusion in a single genus, the presence of internal membranes in *Nitrosolobus* spp. made them sufficiently distinct to be retained as a separate genus. It could, however, be argued that the internal membranes are a single character that distinguish *Nitrosolobus* and *Nitrosospira*. The few other phenotypic data available for these organisms argue for their unification in a single genus. Even morphology of *Nitrosolobus*, *Nitrosospira*, and *Nitrosovibrio* is not a stable character. Spherical cells are produced in cultures of *Nitrosospira* and *Nitrosovibrio*, *Nitrosolobus* is described as pleomorphic [128]. Moreover, a recent study of 16S rRNA sequences from newly isolated *Nitrosospira* spp.

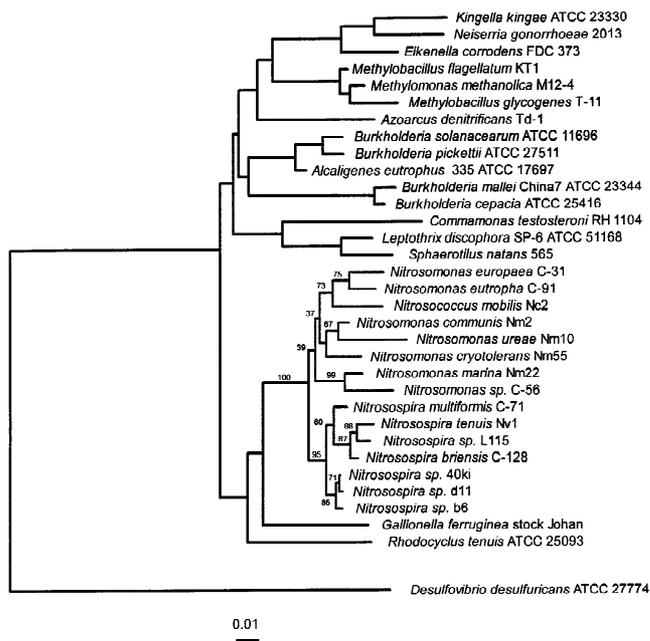


Fig. 5. Phylogenetic relationships among autotrophic ammonia-oxidizing bacteria from the  $\beta$ -Proteobacteria. All sequences used in the analysis were obtained from the RDP except *N. cryotolerans* (Z46948, Z46986), *N. marina* (Z46990, Z46991, Z46992), *N. ureae* (Z46993, Z46994, Z46995), and *N. communis* (Z46981, Z46982, Z46983) which were obtained from the EMBL database (Pommerehne-Roeser, A and Koops, H.-P. Phylogenetic relationships among autotrophic ammonia-oxidizing bacteria, unpublished). Sequences from *Nitrosospira* spp. L115, 40ki, d11, and b6 are from [116].

revealed that the type strain of *Nitrosolobus multiformis* was subsumed within a strongly supported monophyletic group containing all *Nitrosovibrio* and *Nitrosospira* isolates (Fig 5. [116]).

The homology values for 16S rRNA sequences from *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* are 96.9% or greater. This equates to a very small number of nucleotide substitutions, giving low resolution when the taxa are positioned in a phylogenetic tree. Thus, while these ammonia-oxidizers possess distinct morphological differences, these differences seem not to be reflected in the 16S ribosomal DNA sequences. This suggests that the morphological traits have evolved recently, and that 16S ribosomal RNA analysis, alone, is not sufficient to determine the detailed, evolutionary history of these bacteria. The results of the phylogenetic analysis of Utaker and coworkers [116], which showed a low degree of support for *Nitrosolobus* representing a distinct lineage within the  $\beta$ -subdivision autotrophic ammonia-oxidizers, endorse the recent emendation of the genus classification within this group of bacteria [53].

## Molecular Ecological Studies of Autotrophic Ammonia-Oxidizers

The autotrophic ammonia-oxidizing bacteria from the  $\beta$ -subdivision of the Proteobacteria are one of the few monophyletic groups recognizable by rRNA sequence analysis where the physiological properties of the organisms are congruent with the phylogenetic analysis. On the basis of data from cultivated isolates, no organism for which rRNA sequence data are available falls within the  $\beta$ -subdivision ammonia-oxidizer clade that is not an autotrophic ammonia-oxidizing bacterium. Consequently, these ecologically important organisms make ideal candidates for the application of rRNA-based methods in microbial ecology.

A number of environmental studies have stemmed from an increased knowledge of the phylogeny of autotrophic ammonia-oxidizing bacteria. These studies have employed both PCR-based and whole-cell in situ hybridization approaches [58, 61, 77, 79, 118, 122]. In one of these studies, oligonucleotide sequences were selected from the 16S rRNA genes of various species of ammonia-oxidizing bacteria, and evaluated as specific PCR amplification primers and probes. The specificities of primer pairs for eubacterial *Nitrosospira* and *Nitrosomonas* rRNA genes were established with sequence databases. The primer pairs were used to amplify DNA from laboratory cultures and environmental samples. Eubacterial rRNA genes amplified from soil and activated sludge hybridized with an oligonucleotide probe specific for *Nitrosospira* spp., but not with a *Nitrosomonas*-specific probe. Lakewater and sediment samples were analyzed using a nested PCR approach in which eubacterial rRNA genes were subjected to a secondary amplification with *Nitrosomonas* or *Nitrosospira* specific primers. Again, the presence of *Nitrosospira* DNA, but not *Nitrosomonas* DNA, was detected. This was confirmed by hybridization of the amplified DNA with an internal oligonucleotide probe. Enrichments of lakewater and sediment samples, incubated for two weeks in the presence of ammonium, produced nitrite. They also found to contain DNA from both *Nitrosospira* and *Nitrosomonas*, as determined by nested PCR amplification and probing of 16S rRNA genes. This demonstrated that *Nitrosospira* spp. are widespread in the environment, which is further confirmation that those isolates that are easily cultured are not always ecologically dominant [58].

A number of studies have also been conducted with sewage sludge using in situ, whole-cell hybridization with ammonia-oxidizer-specific probes [79, 122]. These showed that the ammonia-oxidizing bacteria were found in dense clus-

ters containing up to 3,000 cells, and, in actively nitrifying plants, up to 20% of the total cells gave a signal with a probe specific for some, but not all *Nitrosomonas* spp.

Dual staining experiments with ammonia-oxidizer-specific probes and a recently developed *Nitrobacter*-specific probe revealed that the nitrite-oxidizing bacteria were associated with the clusters of ammonia-oxidizers found in sewage sludge flocs [79]. In the same study, a wide range of probes was used to detect different taxa of ammonia-oxidizing bacteria. This revealed the presence of cells related to *Nitrosomonas* spp., but *Nitrosospira* was not detected in any of the sewage treatment plants examined. This was also recently observed in a study of nitrifying bacteria from aquarium filters [61]. Interestingly, in the same study, it was noted that samples from freshwater aquaria rarely harbored ammonia-oxidizers from the  $\beta$ -subdivision of the Proteobacteria [61]. Previous studies indicated that *Nitrosospira* was detectable in a range of environments by PCR and probing procedures, while *Nitrosomonas* could be detected only in enrichment cultures from the same samples [58]. It has been suggested that the inability to detect *Nitrosomonas* spp. may have been the result of inefficient DNA extraction from flocs, and that this limitation would not occur with in situ hybridization procedures [79]. This explanation, however, seems unlikely since *Nitrosomonas* could be detected from DNA extracted from enrichment cultures where the cells often exist as flocs. A more likely explanation of a failure to detect *Nitrosomonas* would be that the specificity of the primers used was too great; only on enrichment were low numbers of organisms closely related to *N. europaea* and *N. eutropha* (the organisms that the PCR primers were designed to detect) detected. Furthermore, in situ hybridization studies in our laboratory (T. P. Curtis and I. M. Head, unpublished data) have shown that, while in most nitrifying activated sludge plants sampled, *Nitrosomonas* spp. were dominant, in many cases *Nitrosospira* could be detected. These, too, were present in large aggregates within the sludge flocs. It thus seems likely that the dominant groups of autotrophic ammonia-oxidizing bacteria found in any particular environment will be determined by characteristics specific to that environment.

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## Conclusions and Future Prospects

We have chosen some examples where the application of 16S rRNA PCR sequences analysis and probing with specific fluorescent oligonucleotide probes have generated advances

in microbial ecology in a range of habitats that could not be achieved using conventional techniques alone. However, the role of classical microbial ecology should never be underestimated. With respect to defining a functional role in their particular ecosystems, organisms catalogued only by sequence will permit assessments of diversity only. However, the scope for assigning function to form and to groups of microorganisms remains the challenge of this new technology. The first steps to achieving this are already being made with the application of cloning techniques that allow cloning of large fragments of DNA extracted from environmental samples [107]. This will potentially allow determination of the phylogenetic position of an uncultured organism by sequencing cloned rRNA genes. It will also allow identification of functional genes cloned on the same fragment of DNA, thus giving an indication of the possible metabolic capabilities of an uncultivated organism identified solely from a rRNA sequence.

Combining molecular measures of species composition and the abundance of biogeochemically important groups with measurement of particular processes and environmental parameters is also now being more widely adopted [91, 111]. Such integrated studies will be essential in the future if we are to reap the maximum rewards from nucleic acid-based studies of microbial ecology. Molecular studies are now being complemented by appropriate culture-based investigations [76] that will assist in obtaining cultures of organisms that are truly representative of those important in nature. In addition, where culture is not possible or not an appropriate strategy for the study, quantification of organisms in a particular niche, or an appreciation of their spatial distribution are exciting goals made possible by coupling molecular tools with other developing technologies. For instance, through a combination of confocal microscopy and in situ hybridization, toluene-degrading *Pseudomonas putida* were shown to be distributed throughout a multispecies biofilm. They were found to be active in all regions of the biofilm, although less active than those from batch culture, and responsible for 65% of the toluene degraded by the community [80]. Similarly, the combination of microelectrodes, measuring nitrate concentration, and in situ hybridization to detect nitrifying bacteria has been used to examine their distribution in biofilms. The microelectrodes showed that nitrification occurred in a narrow 50- $\mu$ m zone on top of the biofilm, whereas the ammonia-oxidizers formed a dense layer of cell clusters in the upper part of the biofilm, below which a less dense aggregate of nitrite oxidizers was detected. In addition, both groups were detected in substantially lower

numbers in the anoxic layers deeper in the biofilm [97]. The distribution of sulfate-reducing (SRB) and methanogenic bacteria was also determined in a similar manner, with respect to activity [92]. These examples exemplify the way forward for molecular microbial ecology. In situ hybridization circumvents the need for culture (nitrifying, SRB, and methanogens can be difficult to culture. [92, 97] and, in the case of the toluene degrading biofilm, direct culture of *P. putida* would provide no relevant information [97]); the use of confocal microscopy and microelectrodes provides a means of examination that is relatively noninvasive to the community; and both illustrate the ability to relate community structure to function and activity. Furthermore, the application of amplified ribosomal DNA restriction analysis (ARDRA) may provide a means of examining the succession and convergence/divergence of microbial communities [76]. The laboratory biofilm is, at present, the main focus for these studies, but application of these complementary methods has potential for unravelling the complexities of microbial populations as they exist in nature.

In conclusion, it should be emphasized that molecular ecology is a valuable tool. It should, however, always be appreciated that there are many other equally useful approaches among those used by microbial ecologists. It is only by selecting a range of appropriate tools in a complementary fashion that we can hope to unlock some of the mysteries of microbial ecology.

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