

Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation

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THE CAUSE: FAILURE OF MICROBIOLOGISTS TO DESCRIBE NATURAL DIVERSITY

Among the disciplines devoted to studying the different life forms on our planet, microbiology was the last to be established. Its boundaries are very much defined as a consequence. Subtract from the pool of organisms those that can be studied by the classic techniques of botany and zoology, and the rest is left for microbiologists. What usually remains are small organ-

isms that can be visualized only by using special equipment, microscopes. In contrast to animals and plants, the morphology of microorganisms is in general too simple to serve as a basis for a sound classification and to allow for reliable identification. Thus, until very recently, microbial identification required the isolation of pure cultures (or defined cocultures) followed by testing for multiple physiological and biochemical traits. A successful microbiologist was very much determined by the ability to cultivate microorganisms. Consequently, any approach to identify specific microbial populations without cultivation directly in their natural environments could be revolutionary, since it could change the character of microbiology and close the methodological gap which still exists in comparison with botany and zoology.

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TABLE 1. Culturability determined as a percentage of culturable bacteria in comparison with total cell counts

Habitat	Culturability (%) ^a	Reference(s)
Seawater	0.001–0.1	48, 81, 82
Freshwater	0.25	75
Mesotrophic lake	0.1–1	150
Unpolluted estuarine waters	0.1–3	48
Activated sludge	1–15	160, 161
Sediments	0.25	75
Soil	0.3	153

^a Culturable bacteria are measured as CFU.

The “Great Plate Count Anomaly”

Viable plate count or most-probable-number techniques have been, and frequently still are, used for quantification of active cells in environmental samples. However, because they select for certain organisms, these methods are inadequate to address this problem. Restrictions and potential biases of the established techniques were clearly stated by Brock, who strongly argued in favor of *in situ* studies (21). For oligotrophic to mesotrophic aquatic habitats, it has been frequently reported that direct microscopic counts exceed viable-cell counts by several orders of magnitude. The same is true for sediments and soils (Table 1). Staley and Konopka (150) coined the phrase “great plate count anomaly” to describe this phenomenon, which has been known to microbiologists for generations. By now there is little doubt that in most cases, the majority of microscopically visualized cells are viable but do not form visible colonies on plates (for reviews, see references 123 and 150). Two different types of cells contribute to this silent but active majority: (i) known species for which the applied cultivation conditions are just not suitable or which have entered a nonculturable state and (ii) unknown species that have never been cultured before for lack of suitable methods. It has been well documented for pathogens like *Salmonella enteritidis* (124), *Vibrio cholerae* (28), and *V. vulnificus* (105) that bacteria may quickly enter a nonculturable state upon exposure to salt water, freshwater, or low temperatures.

Even without considering recent molecular data (which will be discussed below), the steady expansion of validly described species reflects the incompleteness of our current knowledge of microbial diversity (24). With the availability of innovative techniques, many more microorganisms will become culturable. One very recent example is the isolation of “ultramicrobacteria” by dilution culture (26, 134). After all, nature can cultivate all extant microorganisms.

Well-Known Uncultured Microorganisms

To be recognized, one has to, first of all, be visible. Second, it helps to look different from the average, to live in a unique location, or to behave or move in a special way. These truisms also hold for microorganisms, as illustrated by the following examples. (i) The intestinal tract of surgeonfish is inhabited by a giant, as yet uncultivated, microorganism that is visible to the naked eye—its individuals being larger than 500 by 80 μm (50). (ii) An economically important symbiosis between microorganisms and vertebrates occurs in ruminant animals (cattle, sheep, etc.), which live on a diet rich in cellulose. The rumen is essentially a large incubation chamber filled with a vast array of morphologically conspicuous microorganisms (176). (iii) Many protozoa contain obligate endosymbionts of bacterial or ar-

chaeal origin. Endonuclear symbionts of ciliates have been known for more than a century (for reviews, see references 64 and 65). Cells of the genus *Holospira* multiply in the macro- or micronucleus of *Paramecium caudatum*. The characteristically elongated (infective) cell forms occurring during the developmental cycle are easily visualized by light microscopy. Anaerobic ciliates live in close association with a variety of endo- and ectosymbionts (46), many of which were tentatively classified as methanogens by the characteristic blue fluorescence of coenzyme F₄₂₀ (158). Nonculturable *Legionella*-like bacteria have been encountered in small amoebae (40, 54). In the giant amoeboid flagellate *Pelomyxa palustris*, several endosymbiotic populations can be distinguished by cell morphology and Gram staining (169). This list could be considerably enlarged, reflecting the common strategy of the unicellular protozoa to allocate certain functions to endosymbionts. Also, many free-living uncultured bacteria have attracted considerable attention. (iv) Bacteria swimming along the lines of a magnetic field have been found in many aquatic and even terrestrial habitats since their initial discovery by Blakemore (for a review, see reference 91). Even though some of these magnetotactic bacteria have been isolated, many more morphotypes have resisted all cultivation attempts. (v) A colorless sulfur bacterium with a size of up to 100 μm and massive CaCO₃ inclusions, *Achromatium oxaliferum*, has tantalized numerous observers, none of whom has, so far, succeeded in developing an effective method of isolation (85).

Despite this quite incomplete listing of well-known uncultured bacteria, one should, however, not forget that most of the hitherto unknown diversity is probably hidden behind inconspicuous cell morphologies. The evolutionarily earliest branching bacterial species, the only recently cultivated hyperthermophile *Aquifex pyrophilus* (73, 107), is just a typical rod.

How Much of the Microbial Diversity Is Currently Known?

From the foregoing, it is obvious that the cultured species of the *Bacteria* and *Archaea* (172) represent only a minor fraction of the existing diversity. Today, about 5,000 species have been described (23), with the *Approved List of Bacterial Names* currently containing less than 3,500 entries. Since the classic species concept for higher organisms applies only to organisms with sexual reproduction, it is very difficult to estimate the number of extant microbial species. For lack of a better alternative, bacterial taxonomists agreed to define a *genospecies* on the basis of a DNA-DNA similarity of more than 70% (131, 165). DNA-DNA reassociation studies on samples directly extracted from soil indicated a complexity comparable to ca. 4,000 completely different standard sizes of bacterial genomes (153). By using the 70% criterion, the authors deduced that there might be as many as 13,000 different species in this sample. Other estimates are based on simplifying calculations like the following. There are at least 800,000 insect species. Each individual harbors millions to billions of bacteria. At least 10% of insect species harbor obligate symbionts, and similar associations occur in ticks and mites (29). Very probably, these are coevolving with their insect hosts (99), which could comprise distinct species. Thus, consideration of insect symbionts alone could increase the number of extant bacterial species by several orders of magnitude. These examples raise the question of what we can do to better understand and access microbial diversity.

POSSIBLE SOLUTION: ANALYSIS OF rRNA MOLECULES

Retrieval of rRNA Sequence Information

The first attempts to characterize environmental samples by studying rRNA began about a decade ago. In these studies, 5S rRNA molecules were directly extracted from mixed samples, the molecules belonging to the different community members were electrophoretically separated, and a comparative sequence analysis yielded phylogenetic placements (84, 148, 149). Three microbial assemblages have been examined by this approach: the bacterial symbionts of "chemoautotrophic invertebrates" that dwell, for example, around deep-sea hydrothermal vents (148), the bacteria inhabiting the 91°C source pool of a hot spring (149), and the microorganisms inhabiting a copper leaching pond (84). These pioneering studies yielded interesting insights. However, the information content in the approximately 120-nucleotide 5S rRNA is relatively small, and the requirement for electrophoretic separation of the different 5S rRNA molecules limited this approach to less complex ecosystems. Consequently, Pace and his associates outlined the use of the larger rRNA molecules for studies in microbial ecology (106). An average bacterial 16S rRNA molecule has a length of 1,500 nucleotides, and 23S rRNA molecules are around 3,000 nucleotides. When fully or almost fully analyzed (at least >1,000 nucleotides should be determined), both molecules contain sufficient information for reliable phylogenetic analyses. The principal steps of the proposed procedure were (i) extraction of total community DNA, (ii) preparation of a shotgun DNA library in bacteriophage lambda, (iii) screening by hybridization with a 16S rRNA-specific probe, (iv) sequence determination from clones containing 16S rRNA genes, and (v) comparative analysis of the retrieved sequences (Fig. 1). The first thorough application of this approach was the characterization of a marine picoplankton sample (133). Numerous unknown sequences could be identified in the library. Among those were 15 unique bacterial sequences related to the cyanobacteria and proteobacteria and 1 eucaryotic sequence.

With the advent of PCR (125), a method became available to speed up this quite laborious procedure. By using PCR, 16S rRNA gene fragments can be selectively amplified from mixed DNA. Gene libraries derived from mixed amplification products should contain only defined fragments that can be rapidly sequenced from known priming sites. This approach was first applied by Giovannoni et al. in an analysis of Sargasso Sea picoplankton. Results indicated again the presence of defined clusters of proteobacterial and cyanobacterial origin (60). This approach greatly reduces or even obviates lengthy screening procedures, which were necessary to identify the rRNA containing clones in shotgun libraries. However, it also bears an additional potential bias for the representative assessment of natural abundance of rRNA genes resulting from the preferential amplification of certain templates. It has been shown that such differential amplification of rRNA genes from mixtures with other rDNAs is not a simply theoretical problem. In the worst case, it can prevent the amplification of certain rDNAs, e.g., those from extremely thermophilic archaea (121); when the DNA was amplified in a DNA mixture containing yeast DNA, the yeast 18S rDNA was preferentially amplified. Archaeal rDNA could be amplified only after addition of 5% (wt/vol) acetamide to the PCR mixture, which minimized non-specific annealing of the primers to yeast rDNA.

Finally, there is yet another route to the molecular characterization of natural microbial communities. Cloning of cDNA transcribed from 16S rRNA with the enzyme reverse transcriptase allows, as in the case of PCR, the selective retrieval of

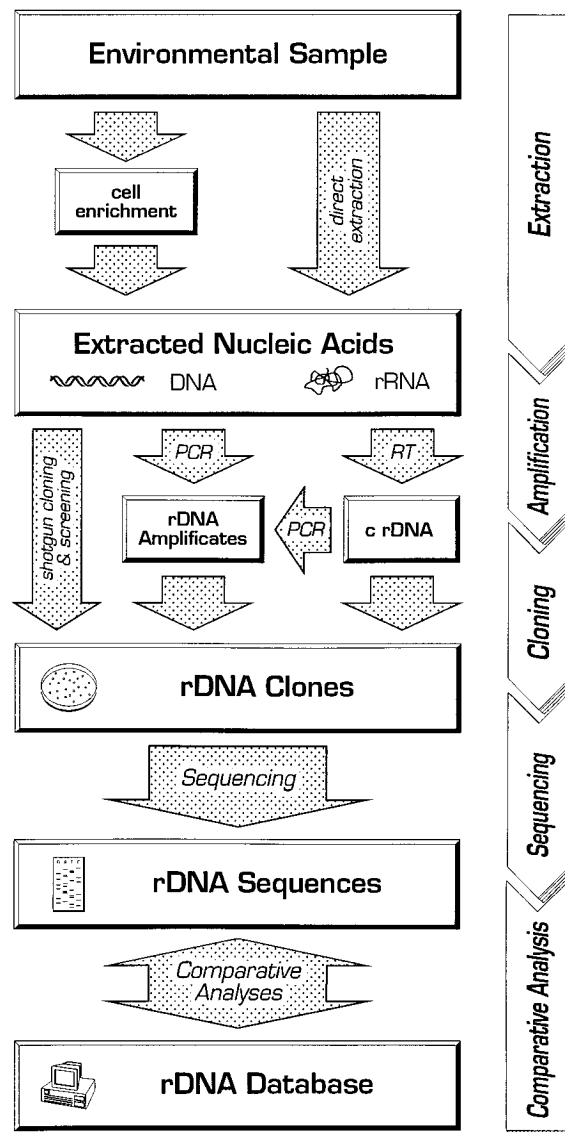


FIG. 1. Flow chart showing the different possibilities to characterize an environmental sample by comparative rRNA sequence analysis. RT, reverse transcriptase. See the text for details. Figure courtesy of M. Bauer.

useful rRNA sequence information (167) (Fig. 1). Ward et al. (164) used this approach to study a cyanobacterial mat growing at 55°C. They were able to discriminate eight distinct sequence types, none of which matched sequences in the 16S rRNA databases derived from about 1,000 cultured bacterial and archaeal species. A variation of this approach involves a PCR step with rDNA-specific primers immediately following the reverse transcription step (5) (Fig. 1). Starting the sequence retrieval from rRNA instead of DNA has the advantages that because of the smaller size of rRNA, more-rigorous nucleic acid extraction techniques (e.g., bead beating [146]) can be applied and a larger number of templates may be available. This procedure might, however, be problematic with respect to the formation of chimeras, a problem which will be discussed below.

The fact that previously unknown sequences were retrieved came as a surprise to many. It indicates that the sequences

deposited in the 16S rRNA databases (86, 103)—mostly culture collection strains, currently encompassing about 25% of the validly described species—do not represent the naturally occurring diversity of microorganisms. It should, however be noted that the culture collections themselves are another source of undescribed diversity. They contain many strains that have been inappropriately named and are currently hidden behind wrong genus or species designations. The current massive sequencing efforts will solve this problem soon. Even when Ward et al. omitted type strains and sequenced isolates obtained from the examined environment, they could not find matches better than 95%.

It is quite difficult to translate rRNA similarity values into divergence in a nomenclatural sense, that is, at the level of a species or genus. As mentioned above, microbial taxonomists currently define a species quite arbitrarily as strains that show 70% or greater DNA-DNA reassociation (165). They also agree that rRNA sequence information alone should not be used to split or lump strains into species. However, comparative studies involving both 16S rRNA sequencing and DNA-DNA hybridization indicated that a 50% DNA-DNA pairing typically corresponds to about 99% 16S rRNA similarity (3). Fox et al. (53) even reported 16S rRNA sequence identity between *Bacillus globisporus* and *B. psychrophilus*, two species that are clearly justified by DNA-DNA similarity of less than 50%. Therefore, these retrieved new rRNA sequences with similarity values below 95% should at least be regarded as good evidence that microbiologists have discovered the presence of novel species.

Designing Probes by Comparative Sequence Analysis

Keeping in mind the number of steps involved in the retrieval of rDNA sequences (Fig. 1), one should be careful to regard these sequence collections as unbiased reflections of the microbial communities living in the natural ecosystems. The initial extraction of nucleic acids is a crucial step since, unfortunately, not all microorganisms lyse equally well. If PCR amplification is involved in the overall procedure, a second severe bias might be introduced. Because of selective priming or higher-order structural elements, certain sequences may be discriminated slightly compared with others. This would result in huge differences after multiple cycles. It is also important to check for contamination, a problem commonly encountered with PCR. Another problem, the formation of chimeric PCR products, will be discussed later. Third, biases can occur during the cloning step. Care has to be taken, e.g., by the use of rare-cutting restriction enzymes like *NotI*, that restriction enzymes applied in forced cloning approaches must not cut within certain amplified rDNA fragments. Furthermore, different cloning efficiencies for rRNA gene fragments from different organisms cannot be ruled out. Besides these technical points that can eventually be addressed, quantification of the relative abundance of certain populations from the relative abundance of certain rDNA clones will always be biased by the fact that the number of rRNA gene operons present in a bacterial chromosome can range from 1 copy (mycoplasmas [7]) to up to 10 copies (bacilli [74]).

It is therefore important to corroborate the presence and to estimate abundance of the retrieved sequences within the initially extracted nucleic acids, within the mixed pool of amplification products, or preferentially within the cells present in the original environmental sample. This can best be achieved by hybridization with nucleic acid probes as indicated in Fig. 2. The design of such probes has been reviewed in detail recently (144). The principal steps are the alignment of rRNA (gene)

sequences, the identification of sequence idiosyncrasies, the synthesis and labeling of complementary nucleic acid probes, and finally the experimental evaluation and optimization of the probe specificities and assay sensitivities. Our knowledge of the microorganisms and sequences present in a certain ecosystem is obviously limited by the number of sequences that have been retrieved and determined. The thorough knowledge of the 16S and 23S rRNA conservation profiles does, however, allow the investigator to direct a probe to a highly variable target site. A probe, intended to be specific for a unique sequence, has to be compared to all accessible rRNA sequences. It must also be evaluated by hybridization against cultured reference strains (35, 92). Although unlikely, even then, it cannot be ruled out that more than one species will hybridize to the "specific" probe. It should now be clear that the sequence database should be representative of the expected complexity of the sample to increase the probability that a certain 18-mer oligonucleotide probe will quantify the abundance of a defined 16S rRNA sequence (and thereby a certain microbial population) and not just the abundance of the target site. On the other hand, if the goal of a study is to monitor the fate not of a narrowly defined taxonomic unit but of a broader group of microorganisms, probes can be tailored for this purposes by targeting less variable sites on the rRNA molecule.

Quantitative Dot Blot Hybridization

There are two different ways to use phylogenetically based oligonucleotide probes for quantification. Quantification of a certain 16S rRNA compared with total 16S rRNA can be obtained by dot blot hybridizations of a directly isolated nucleic acid mixture with universal and specific oligonucleotide probes. The relative abundance is calculated by dividing the amount of specific probe bound to a given sample by the amount of hybridized universal probe (measured, e.g., as counts per minute for radioactively labeled probes). In a ground-breaking study, this approach was first applied to the monitoring of population changes in the rumens of cattle (146). In this strictly anaerobic environment, virtually all microorganisms are fastidious and difficult to grow. Upon addition of the antibiotic monensin to the animals' diet, pronounced population changes could be observed for different *Fibrobacter* (sub)species. When radioactively labeled oligonucleotide probes were used in a dot blot assay, rRNA sequences with a relatively low abundance between 0.1 and 1% could be quantified. One should be aware that these data of relative rRNA abundance can not be directly translated into cell numbers. Cells of different species have different ribosome contents ranging roughly between 10^3 and 10^5 ribosomes per cell. Even for one strain, cellular rRNA contents can vary significantly (at least over one order of magnitude), since they are directly correlated with the growth rate (33, 78, 127, 162). The relative rRNA abundance should, however, represent a reasonable measurement of the relative physiological activity of the respective population, since it is the product of the number of detected cells and the average rRNA content. This information on the general activity of a given population should not automatically be regarded as an indication of a specific kind of activity. Often, one population has the potential to catalyze different transformations—one genotype is linked to several phenotypes.

Whole-Cell and In Situ Hybridization

In situ identification and enumeration of microorganisms harboring a certain rRNA sequence require a technique in which the rRNA is specifically detected within morphologically

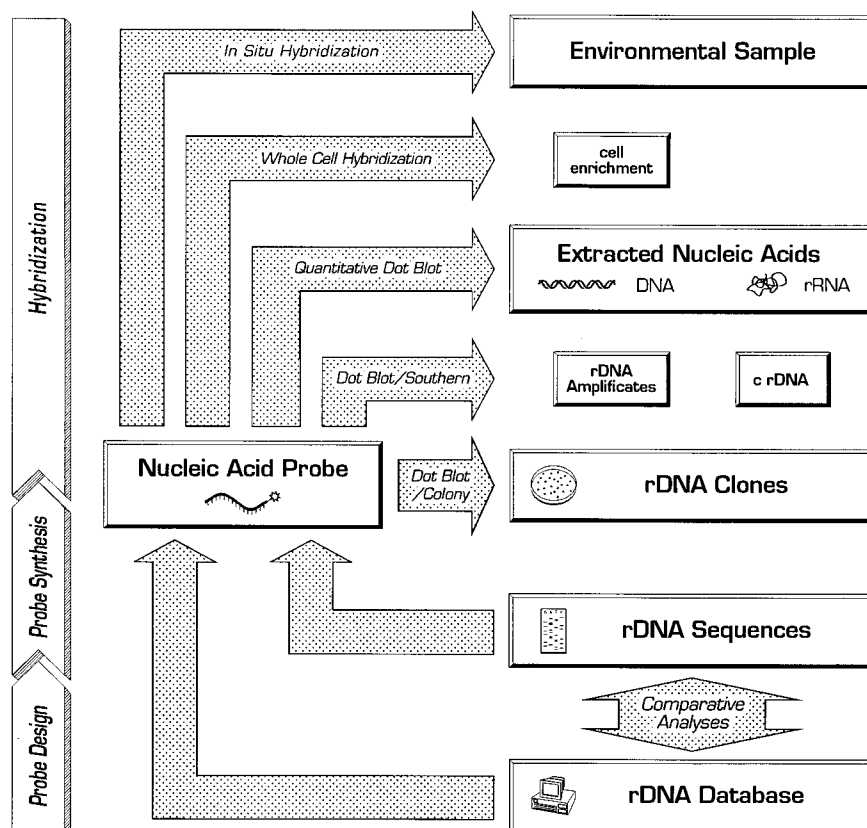


FIG. 2. Flow chart showing the different options of using rRNA-targeted nucleic acid probes to characterize an environmental sample by hybridization techniques. See the text for details. Figure courtesy of M. Bauer.

intact cells, a technique here referred to as whole-cell hybridization. The more common term "in situ hybridization" will be restricted in this review to whole-cell hybridizations in which cells are detected in their natural microhabitat. Analysis at the single-cell level by whole-cell hybridization can obviously provide a more detailed picture than dot blot hybridization. One can not only determine the cell morphology of an uncultured microorganism and its abundance but also analyze spatial distributions in situ. Quantification of the signal conferred by rRNA-targeted oligonucleotides should even allow the estimation of in situ growth rates of individual cells (111).

The use of in situ hybridization for counting and identifying organisms had already been proposed by Olsen et al. (106). The microscopic identification of single microbial cells with rRNA-targeted probes was first performed with radioactively labeled oligonucleotides (61). After hybridization of whole fixed cells, the ^{35}S -labeled probe was visualized by microautoradiography. This step not only required extra time but also resulted in formation of silver grains up to several micrometers away from the target cell, thus preventing the exact in situ localization of small cells in clusters. Although this problem could be improved by using less energetic isotopic labels like ^3H , the trade-off for a 1 μm -resolution would be an extension of the time required for autoradiography to several weeks or months.

As demonstrated several years ago by the immunofluorescence approach (for a review, see reference 17), fluorescent probes yield superb spatial resolution and can be instantaneously detected by epifluorescence microscopy. Fluorescently monolabeled, rRNA-targeted oligonucleotide probes were

demonstrated to allow detection of individual cells (33). This made whole-cell hybridization with rRNA-targeted probes a suitable tool for determinative, phylogenetic, and environmental studies in microbiology (2). Like immunofluorescence, whole-cell hybridization with fluorescently labeled rRNA-targeted oligonucleotides can be combined with flow cytometry for a high-resolution automated analysis of mixed microbial populations (1a, 162).

Closing In with Nested Probes (Top-to-Bottom Approach)

The rRNA approach to the phylogenetic identification and in situ detection of microorganisms is often used in a sequential manner in which the samples are first characterized by the retrieval of rDNA sequences. In the second phase, the abundance of certain clones, specific rRNA sequences, or sequence-harboring cells is quantified by using highly specific oligonucleotide probes in a colony, dot blot, or whole-cell hybridization (Fig. 3). Although PCR-assisted approaches are very powerful, the analysis of complex communities remains rather laborious and time-consuming, forcing most researchers to cut down on the number of examined clones. Even when several hundred clones are examined, smaller populations (around or below 1%) may not be detected. Therefore, there is a need for an alternative path to a complete molecular characterization of environmental samples. It is well established that on various taxonomic levels, phylogenetic groups are characterized by regional rRNA sequence idiosyncrasies, their so-called signatures. New isolates can be assigned to, e.g., one of the major bacterial taxa by signature analysis (175). Oligonucleotide

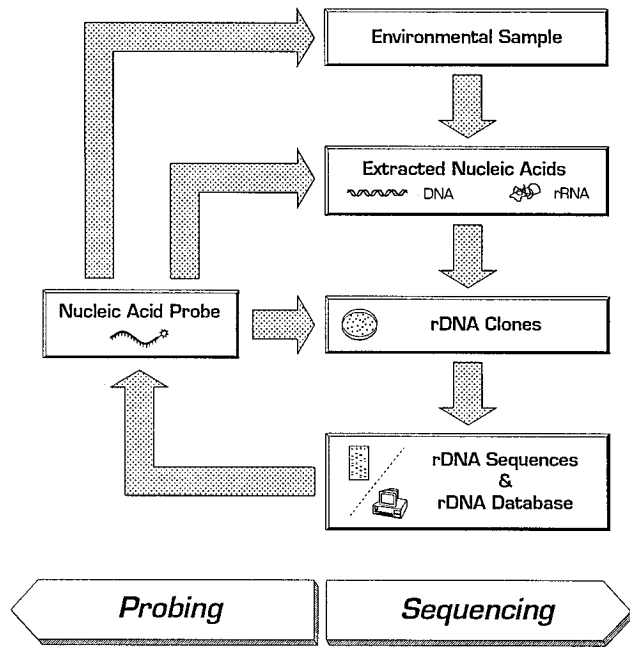


FIG. 3. Flow chart showing the principal phases of the rRNA approach for sequencing and probing. Figure courtesy of M. Bauer.

probes are more suitable for routine screening of such signatures than is sequencing (61, 142, 143, 146). Such probes have been designed for the highest taxonomic levels, the domains *Archaea*, *Bacteria*, and *Eucarya* (2, 33, 61, 144); for intermediate levels, e.g., the gram-negative sulfate-reducing bacteria (5), the alpha, beta, and gamma subclasses of *Proteobacteria* (92), the flavobacterium-cytophaga and bacteroides clusters (93) of the CFB phylum (170), the two archaeal kingdoms *Crenarchaeota* and *Euryarchaeota* (25), the orders *Methanobacteriales* and *Methanococcales* of the methanogens (114); and for lower taxa (genus-, species-, and subspecies-specific probes) (see, e.g., references 3, 35, 63, 126, 146, 161). When such probes are applied to parallel subsamples in an ordered top-to-bottom approach, initially using a universal and three domain-specific probes followed by probes of more and more narrow specificity, increasingly refined information on the community diversity and composition can be obtained very rapidly by both dot blot (see, e.g., reference 113) and whole-cell (95, 160) hybridization. In this approach, the information gained from the higher-level probes are used to select the probe sets tailored for the next lower taxonomic level; e.g., if the domain-specific probing reveals dominance of *Archaea*, probes discriminating between the two groups of *Crenarchaeota* and *Euryarchaeota* (25) are applied in the next step before probing for the major orders of *Euryarchaeota* (114). Several probes have been designed to monitor larger phylogenetic groups within the *Bacteria* and *Archaea* (Fig. 4). Here it is time for another disclaimer. Currently, group-specific probes are designed almost completely on the basis of sequences of cultured organisms and their specificities are evaluated by hybrid-

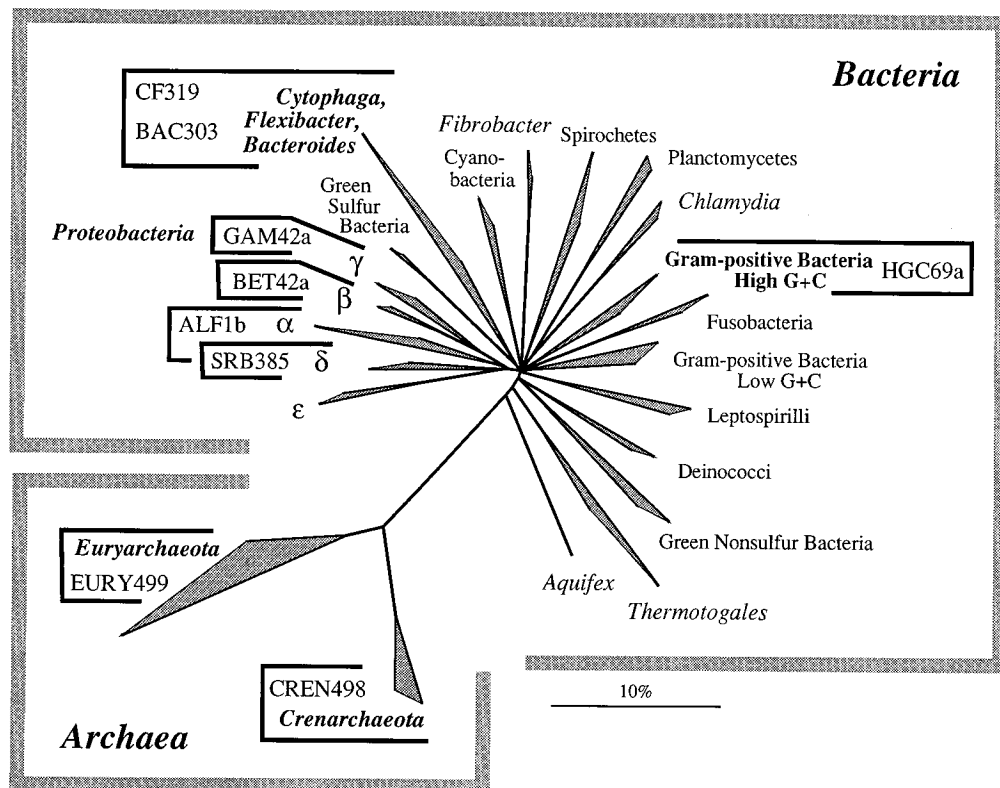


FIG. 4. Phylogenetic tree (based on a dataset of about 1,800 bacterial and archaeal 16S rRNAs) showing the branches currently targeted by upper group-level oligonucleotide probes (brackets; for the respective probe sequences, see Table 3). The phylogenetic depth in the major lines of descent is indicated by the dotted triangles. The bar corresponds to 10% estimated sequence divergence.

TABLE 2. Bacterial and archaeal symbionts of protozoa, insects, and invertebrates analyzed by the rRNA approach

Host	Symbiont	Phylogenetic affiliation of symbiont	Reference(s)
Invertebrates			
<i>Riftia pachyptila</i> (tube worm)	Chemoautotrophic endosymbiont in trophosome	Gamma subclass of <i>Proteobacteria</i>	38, 148
<i>Calypotgena</i> sp. (bivalve)	Chemoautotrophic endosymbiont in gill tissue	Gamma subclass of <i>Proteobacteria</i>	27, 38, 148
<i>Thyasira flexuosa</i> (bivalve)	Chemoautotrophic endosymbiont in gill tissue	Gamma subclass of <i>Proteobacteria</i>	39
<i>Lyrodus pedicellatus</i> (bivalve; shipworm)	Cellulolytic endosymbiont in gill tissue	Gamma subclass of <i>Proteobacteria</i>	37
<i>Laxus</i> sp. (formerly <i>Catanema</i> sp.)	Chemoautotrophic ectosymbiont	Gamma subclass of <i>Proteobacteria</i>	109
Insects			
Aphids	<i>Buchnera aphidicola</i> (primary symbiont)	Gamma subclass of <i>Proteobacteria</i>	98, 100
	Secondary endosymbiont	Gamma subclass of <i>Proteobacteria</i>	157
Hymenoptera	<i>Wolbachia pipientis</i> , endosymbiont causing sex ratio distortion	Alpha subclass of <i>Proteobacteria</i>	108
			151
<i>Adalia bipunctata</i> (ladybird beetle)	Male-killing endosymbiont	Alpha subclass of <i>Proteobacteria</i>	168
Protozoa			
<i>Paramecium caudatum</i>	<i>Holospora obtusa</i> , <i>H. elegans</i>	Alpha subclass of <i>Proteobacteria</i>	4
<i>Paramecium caudatum</i>	<i>Caedibacter caryophila</i>	Alpha subclass of <i>Proteobacteria</i>	139
<i>Euplotes aediculatus</i>	<i>Polynucleobacter necessarius</i>	Beta subclass of <i>Proteobacteria</i>	138
<i>Acanthamoeba castellanii</i>	<i>Sarcobium lyticum</i>	Gamma subclass of <i>Proteobacteria</i>	140
<i>Metopus contortus</i>	Methanogenic endosymbiont	<i>Methanobacteriales</i>	43
<i>Metopus palaeoformis</i>	Methanogenic endosymbiont	<i>Methanobacteriales</i>	44
<i>Trimyema</i> sp.	Methanogenic endosymbiont	<i>Methanobacteriales</i>	49
<i>Pelomyxa palustris</i>	Thin methanogenic rod	<i>Methanobacteriales</i>	138
	Thick endosymbiont	<i>Methanomicrobiales</i>	138
<i>Metopus contortus</i>	Sulfate-reducing ectosymbiont	Delta subclass of <i>Proteobacteria</i>	47

ization against reference organisms (35, 92). This is an obvious drawback, since the cultured microorganisms probably represent only a minor fraction of the real diversity of organisms and sequences. The situation will change with the integration of directly retrieved rDNA sequences into the public databases.

Putting It All Together: the rRNA Approach

Now, all the tools that are necessary for an identification and phylogenetic characterization of microorganisms without cultivation have been explained. As outlined in Fig. 1, one can start with the extraction of DNA from an environmental sample, use standard molecular techniques to obtain a clone library and to retrieve rDNA sequence information, and perform a comparative analysis of the retrieved sequences. This yields information on the identity or relatedness of new sequences in comparison with the available databases and gives a minimal estimate of the genetic diversity in the examined sample. However, this is not proof that the retrieved sequences were from cells thriving in this habitat. They could also have originated from naked DNA present in the sample or from contamination. Therefore, in the second phase (Fig. 2), sequence-specific hybridization probes have to be designed to identify and enumerate whole fixed cells in the original sample by in situ hybridization. Since this step brings us back to the starting material, we have closed a cycle and are consequently referring to this biphasic approach as "full-cycle rRNA analysis." Often, it is also informative to quantify the abundance of a certain rRNA or rDNA in the extracted nucleic acid pool by dot blot hybridization or of a certain clone in the library by colony hybridization. Only by a combination of probing and sequencing (Fig. 3) can one fully exploit the potential of this approach. The rRNA approach offers a cultivation-independent alternative to the established techniques for the identification of new microorganisms. It should, however, be stressed that even a complete rRNA study cannot substitute for the isolation and characterization of microorganisms. Certain as-

pects of the rRNA analysis of microorganisms in natural samples have recently been reviewed by Ward et al. (163).

APPLICATIONS OF THE rRNA APPROACH

Not surprisingly, initial applications of the rRNA approach focused on identification of microorganisms in less complex samples. Once its suitability had been demonstrated, it started to find widespread application. Consequently, in this section we present an overview on some of these studies, going from less to more complex environments.

Currently, only part of the applications of the rRNA approach encompass sequencing and probing. To allow a reasonably complete review, the following compilation also includes studies based solely on either one of the two principal parts of the rRNA approach.

Symbiotic Bacteria and Archaea

Microbial symbionts form a quite prominent fraction of hitherto uncultured microorganisms. They live in continued close association with other organisms that can be categorized as obligate or facultative, mutualistic or parasitic, and ecto- or endosymbioses. Although many symbionts have been named solely on the basis of morphological criteria, their taxonomic position is essentially unknown (29). From a microbial perspective, the host organism can be regarded as a small ecosystem that is inhabited only by a few, often only one, well-adapted species. From a microbiologist's standpoint, such samples have a limited complexity and are ideally suited for testing new techniques such as the rRNA approach (Table 2).

Chemoautotrophic invertebrates. Certain invertebrates like the tube worm *Riftia pachyptila* and clams of the genus *Calypotgena* dwell in the environments surrounding hydrothermal vents and are characterized by the maintenance of large numbers of sulfur-oxidizing microorganisms in specialized tissues. These obligate symbioses form the so-called chemoautotrophic animals. Stable carbon isotopic analyses have revealed identi-

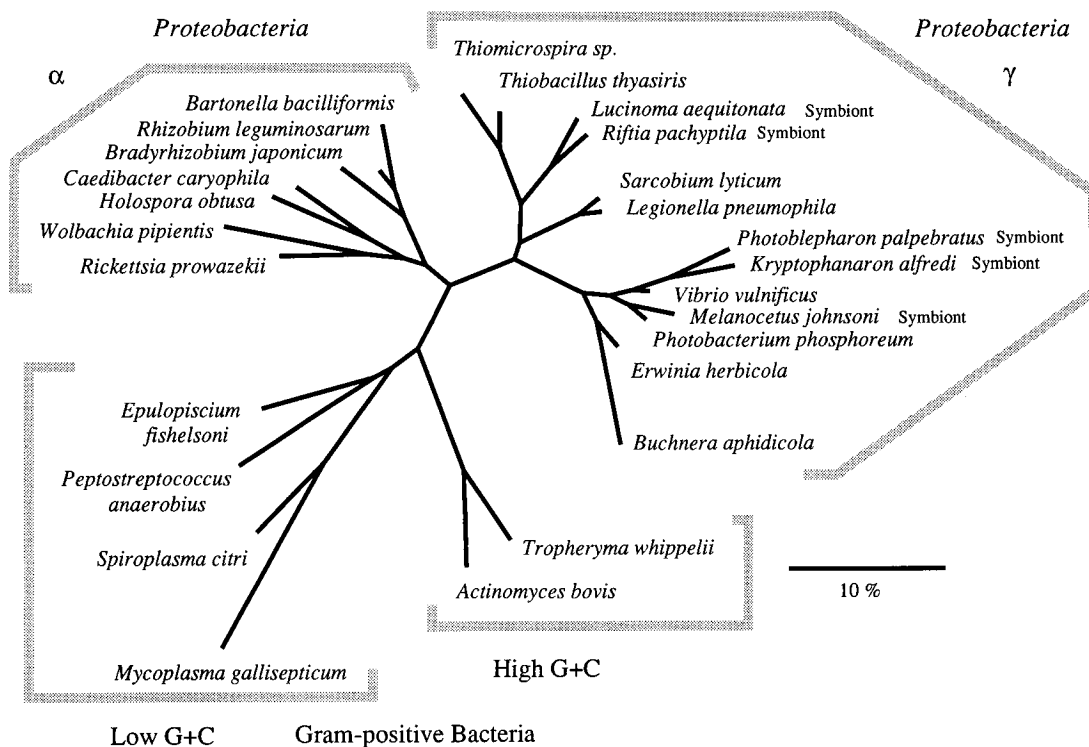


FIG. 5. Phylogenetic tree showing the affiliations of several endosymbiotic bacteria. The bar indicates 10% estimated sequence divergence.

cal $^{12}\text{C}/^{13}\text{C}$ ratios for hosts and endosymbionts and the depletion in ^{13}C characteristic for chemoautotrophic bacteria. The hosts rely for their nutrition on the chemoautotrophy of the symbiont. Since characterization of symbionts was hampered by their resistance to cultivation, the rRNA approach was taken (148). This study represents the first application of the approach to uncultured symbionts. Extraction of tissue samples and electrophoretic separation of 5S rRNAs yielded only two dominant bands from each invertebrate examined, one from the host and one from the symbiont. By sequencing, the directly retrieved 5S rRNA molecules from the symbionts were shown to be related to the gamma subclass of *Proteobacteria*. Later, by analysis of partial 16S rRNA sequences, this affiliation was corroborated, and it was shown that all symbionts cluster in a part of the gamma subclass of *Proteobacteria* that also encompasses free-living sulfur-oxidizing bacteria (Fig. 5) (38). An analysis of 16S rDNA fragments and in situ hybridization with rRNA-targeted oligonucleotide probes proved the transovarian inheritance of the endosymbiotic bacteria in *Caelyptogena* spp. (27). Characterization of the gill symbiont of the bivalve *Thyasira flexuosa* by PCR and 16S rRNA sequence analysis revealed that the symbiont was not identical to a previously reported putative symbiont isolate, *Thiomicrospira thyasiris* (39). Whereas the rRNA of the isolate resembles that of a free-living chemolithoautotrophic bacterium, the bacterial 16S rDNA amplified from the gill tissue was most closely related to the 16S rRNA of known symbionts of clams (Fig. 5). In a similar case, oligonucleotide hybridization provided direct visual evidence that cellulolytic nitrogen-fixing bacteria, isolated from shipworms, wood-boring molluscs of the family *Teredinidae* (Bivalvia), were indeed the symbionts observed in the gill tissues. They enable their host to utilize wood as principal food source (37). By applying the rRNA approach, the bacterial ectosymbionts covering free-living marine nematodes

(Stilbonematinae) were shown to be closely related to the sulfur-oxidizing endosymbionts (109). Here, the host has a digestive tract and apparently grazes on its microbial coat. By active motion of the host, the bacteria are held in an optimal chemical environment within the gradients of oxygen and reduced-sulfur compounds that are present in the sediments.

Endosymbiotic bacteria in insects. Since obligate intracellular symbioses between bacteria and insects are extremely widespread (29), we will focus here on only a few well-studied associations. The maternally inherited primary endosymbionts of aphids live in specialized host cells, the mycetocytes. In its active stage of growth, an aphid contains approximately 4×10^6 endosymbionts in 70 mycetocytes (13). Since aphids feed solely on plant juices, they are probably dependent on essential amino acids produced by their primary endosymbionts (which have not been grown away from their hosts). Phylogenetic analyses of 16S rDNA sequences revealed that the primary endosymbionts form a monophyletic group within the gamma subclass of *Proteobacteria* (Fig. 5) and were probably coevolving with their hosts (99, 157). This lineage was subsequently named *Buchnera aphidicola* (100). The molecular rRNA clock of the endosymbiotic bacteria was calibrated on data inferred from fossil aphids and suggested that long-term cospeciation occurred in the aphid-bacterium symbiosis (98). Since certain aphids also contain secondary endosymbionts associated with sheath cells of the mycetomes, it is somewhat unsatisfying that only indirect evidence (strength of restriction fragment length polymorphisms) was used to assign two retrieved sequences to the primary and secondary endosymbionts (157). In situ hybridization with sequence-specific probes would offer a straightforward way to corroborate these assignments.

Bacterial endosymbionts are also involved in cytoplasmic incompatibility in insects. This term describes the phenomenon in which certain crosses between symbiont-infected individuals

lead to embryonic death or sex ratio distortion toward the haploid sex. PCR-assisted sequencing of 16S rDNA genes directly from infected insect tissues revealed that the symbionts belong to the alpha subclass of the *Proteobacteria* (108). On the basis of morphology and high sequence similarity, all symbionts were assigned to the species *Wolbachia pipientis*. The nearest relatives of *W. pipientis* are arthropod-borne pathogens of mammals, such as *Anaplasma marginale*, *Ehrlichia risticii*, and the *Rickettsia* spp. (Fig. 5). This type of insect-bacterium symbiosis seems to be quite widespread. Closely related sequences (99% similarity) could be retrieved from microorganisms associated with the parthenogenesis of parasitoid *Hymenoptera* (151) and with male killing in the ladybird beetle *Adalia bipunctata* (168). In contrast to the findings for the aphid symbionts, no congruence between the phylogeny of the symbionts and their insect hosts (coevolution) was evident, indicating horizontal transfer of symbionts between insect species (108).

Bacterial and archaeal symbionts of protozoa. Full-cycle rRNA analyses were performed for the symbiotic bacteria of the genus *Holospora* which live in the nuclei of paramecia (4). *Holospora elegans* and *H. undulata* infect micronuclei of *Paramecium caudatum*, whereas *H. obtusa* infects the macronucleus in other strains of the same host species. Here the situation was complicated by the fact that these ciliates also feed on bacteria, so that bacterial rRNA can be detected not only in the nuclei but also in the food vacuoles of the host (Fig. 6). After amplification, cloning, and sequencing of rDNA gene fragments, complementary oligonucleotide probes were used to unambiguously assign the newly retrieved sequences to the intranuclear symbionts of *P. caudatum*. Consequently, the phylogenetic position of *H. obtusa* and *H. elegans* and not just of "sequences retrieved from infected paramecia" could be verified. The genus *Holospora* groups in the alpha subclass of *Proteobacteria* and is most closely related to another endosymbiont of *P. caudatum*, *Caedibacter caryophila* (139). This bacterium, like the other members of the genus *Caedibacter*, is toxic to susceptible strains of paramecia and thereby confers a killer trait to its host. Interestingly, the 16S rDNA of this hitherto uncultured bacterium contains an unusual insertion of 194 nucleotides at ca. position 186 (*Escherichia coli* numbering [22]). The intervening sequence is not present in the fragmented mature 16S rRNA of *C. caryophila* (139). Phylogenetically, these endosymbionts of paramecia show a distinct relatedness to intracellular parasites of the genus *Rickettsia* (Fig. 5).

Prokaryotic endosymbionts are also quite abundant in other single-celled protozoa (Table 2), where they fulfill a variety of tasks for their hosts. Other examples are the methanogenic symbionts of anaerobic ciliates. The polymorphic cells in the host *Metopus contortus* were shown by full-cycle 16S rRNA analyses to contain identical rRNAs, closely related to the archaeon *Methanococcus parvum* (43). This endosymbiotic species is intriguing in showing a morphological transformation accompanied by a partial loss of its cell wall. The resulting enlarged cells attach to host organelles which generate hydrogen (hydrogenosomes). The authors speculate that for large ciliates like *M. contortus*, the intracellular hydrogen concentration could become inhibitory. This would be prevented by the hydrogen-utilizing intracellular methanogens. Here, the rRNA approach not only proved the identity of the polymorphic cells but also demonstrated that a methanogen previously isolated from a culture of *M. contortus* was unrelated to the hydrogenosome-associated cells. In similar studies, the phylogenetic positions of the methanogenic endosymbionts of *M. palaeoformis* and *Trimyema* sp., two other anaerobic ciliates, were analyzed (44, 49).

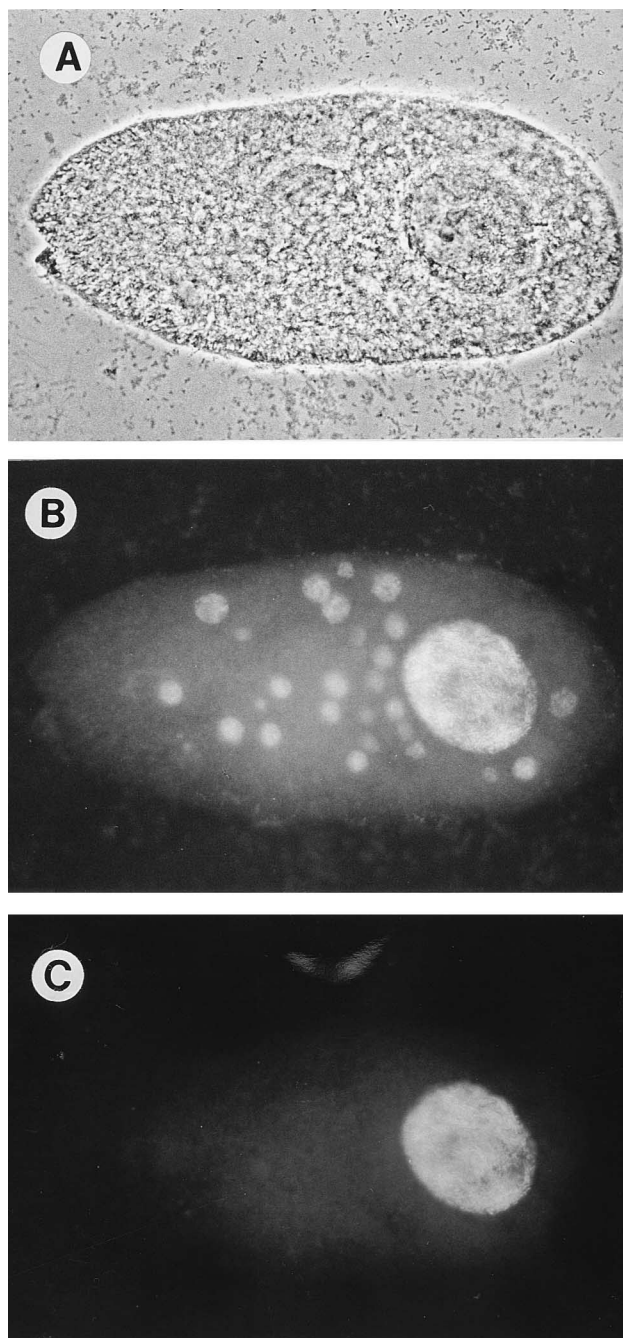


FIG. 6. In situ hybridization of a paraformaldehyde-fixed *Paramecium caudatum* cell with probes specific for the domain *Bacteria* (labeled with fluorescein) and the genus *Holospora* (labeled with tetramethylrhodamine). Phase-contrast micrographs (A) and epifluorescence micrographs (B [using a fluorescein-specific filter set] and C [using a rhodamine-specific filter set]) were taken from one microscopic field. Whereas the fluorescein conferred by the bacterial probe can be detected in both food vacuoles and the macronucleus, rhodamine-stained cells of *Holospora obtusa* can be visualized only in the macronucleus. Photomicrographs by N. Springer.

The giant amoeboid flagellate *Pelomyxa palustris* contains two morphologically distinct populations (thick and thin rods) of cytoplasmic endosymbionts. Whereas the thin rods had been identified as methanogenic archaea on the basis of the presence of the deazaflavin coenzyme F₄₂₀ and the pterin com-

pound F₃₄₂ (158), no characteristic fluorescence was detectable for the thick rods. By *in situ* hybridization (138) with a domain-specific oligonucleotide probe (Arch915 [144]), both rod-shaped morphotypes were identified as archaea. Of the two different retrieved sequences, one was closely related to *Methanosaeta concilii* (formerly "*Methanothrix soehngenii*" and "*Methanothrix concilii*"). It could be assigned to the thick rods by specific oligonucleotide probing. The 16S rRNA sequence of the small rod was highly similar to *Methanobacterium bryantii* (95% similarity). Interestingly this is the first example in which members from two archaeal orders, the *Methanobacteriales* and *Methanomicrobiales*, form a stable endosymbioses with their protozoal host.

Other recent applications of the rRNA approach have yielded evidence that symbiotic life is not restricted to the alpha subclass of *Proteobacteria* or methanogens. *Sarcobium lyticum*, an obligate intracellular parasite of small amoebae, phylogenetically has to be regarded as a *Legionella* species (140) belonging to the gamma subclass of *Proteobacteria* (Fig. 5). *Polynucleobacter necessarius*, a gram-negative obligate symbiont in the cytoplasm of the ciliate *Euplotes aediculatus* (71), was demonstrated to be affiliated with the beta subclass of *Proteobacteria* and to be most closely related to *Alcaligenes eutrophus* (138). By using a group-specific 16S rRNA-targeted oligonucleotide, ectosymbiotic bacteria covering certain marine, free-living anaerobic ciliates (*Metopus contortus*, *Caenomorpha levanderi*) were shown to be gram-negative sulfate-reducing bacteria belonging to the delta subclass of *Proteobacteria* (47). The diversity of such endosymbiotic relationships is also documented in the occurrence of purple nonsulfur bacteria in the marine ciliate *Strombidium purpureum*. The resulting symbiosis requires light for survival and growth under anaerobic conditions and seems to be the first example of a symbiotic relationship between nonoxygenic phototrophic bacteria and eucaryotes (45). This finding supports speculations about the origin of mitochondria. rRNA sequence data indicate (177) that mitochondria could have evolved from endosymbionts belonging to the phototrophic nonsulfur bacteria within the alpha subclass of *Proteobacteria*.

Symbioses between vertebrates and bacteria. Prominent examples of mutualistic symbiotic associations between vertebrates and bacteria are the light organs of fish. Such bioluminescent symbioses can range from facultative ones with culturable *Photobacterium* spp. to highly adapted, apparently obligate ones between hitherto noncultivable bacteria and flashlight fish or deep-sea anglerfish. Haygood and Distel (70) have shown by 16S rRNA analyses that these endosymbionts form a unique lineage related to the genus *Vibrio* (Fig. 5). They are characterized by host specificity, deep divergence between the symbionts from the anglerfish and the flashlight fish, and, possibly, coevolution of host and symbionts.

Despite living inside their hosts, the microorganisms dwelling in gastrointestinal tracts of animals should be regarded as ectosymbionts. Although many of these microorganisms are facultative symbionts that can be readily cultivated outside their hosts, others have not been cultivated (176). Species- and group-specific 16S rRNA-targeted oligonucleotides were used to monitor *Fibrobacter* spp. and *Lachnospira multiparus* in bovine rumen samples before, during, and after experimental perturbation of the ecosystem (146). A recent study applying full-cycle rRNA analysis revealed that the huge microorganisms (*Epulopiscium fishelsoni*) inhabiting the intestines of surgeonfish are currently the largest known bacteria. Comparative sequence analysis of clones obtained from PCR-amplified 16S rDNA fragments indicated an affiliation to the group of gram-positive bacteria with a low-GC DNA content (Fig. 5). *In situ*

hybridization with two specific oligonucleotides proved that the rDNA clones were derived from the symbionts and not from other members of the intestinal microbiota or contaminants. The strength and even distribution of the probe-conferred fluorescence also demonstrated high cellular ribosome content and little subcellular compartmentalization (8).

Identification of pathogens. Clinicians have long been aware of human diseases that are associated with visible but nonculturable microorganisms. Several studies have used the rRNA approach to identify the causative agents. One example is the work on Whipple's disease, in which a "bacillus" infection of the duodenum and small intestine causes a fever, abdominal pain, and diarrhea. On the basis of 16S rDNA analyses, the Whipple's-disease-associated rod-shaped bacterium was identified as a previously uncharacterized actinomycete (Fig. 5), not closely related to any known genus (120, 170), for which the name "*Tropheryma whippelii*" has been proposed (120).

Also, the silver-staining cells seen within the lesions of bacillary angiomatosis, assumed to be noncultivable, have been examined by the rRNA approach (118, 119). The 16S rDNA sequence analysis revealed a close similarity to the genera *Bartonella* and *Rochalimaea*, a group of usually intracellular, often pathogenic bacteria within the alpha subclass of *Proteobacteria*. Near identity of the amplified sequence with the 16S rDNA of the recently isolated *Rochalimaea henselae* (99.8% [116, 119]) makes it very likely that this organism is the causative agent of bacillary angiomatosis. Brenner et al. (19) proposed, on the basis of DNA hybridization, to unify the genera *Bartonella* and *Rochalimaea* and to rename *R. henselae* as *Bartonella henselae* comb. nov. Furthermore, it was suggested to remove the family *Bartonellaceae*, whose members are cultivable on bacteriological media, from the order *Rickettsiales*, which should contain only obligately intracellular bacteria. This is an indication that at least some of the microorganisms now assumed noncultivable will eventually be grown in pure culture.

Other studies also encompassed the identification of microorganisms in clinical samples. *Pneumocystis carinii* is an opportunistic pathogen that poses a considerable threat to immunodeficient patients. After the 16S-like rDNA was sequenced, three biotin-labeled oligonucleotides corresponding to the regions with the least sequence similarity with other rRNAs were used to unequivocally establish that a retrieved sequence originated in *P. carinii* cells (41). The comparative sequence analysis demonstrated that *P. carinii* is a member of the fungi. *In situ* fluorescence hybridization enabled the identification of gram-negative anaerobes like *Bacteroides forsythus* in subgingival plaque samples from patients with advanced periodontitis (58, 59). Molecular techniques will probably become routine methods not only for the detection of "nonculturable pathogens" but also for those cases in which they allow more rapid diagnosis than conventional cultivation techniques. Consequently, there have been several studies on the identification of slowly growing pathogens such as the members of the genus *Mycobacterium*; e.g., a species-specific assessment of *Mycobacterium leprae* in skin biopsy specimens by *in situ* hybridization with a rRNA-targeted oligonucleotide has been reported (9).

The same techniques have also been used to analyze animal and plant pathogens. An obligate intracellular bacterium of porcine intestines was recently described as *IS* (ileal symbiont) *intracellularis* (57). The gram-negative, nonflagellated, curved rod is located freely within the cytoplasm of epithelial cells in the ilea of pigs and is probably the cause of a proliferative enteritis. 16S rDNA sequence analysis indicated its affiliation with the delta subclass of *Proteobacteria*, with 91% similarity to *Desulfovibrio desulfuricans* (57).

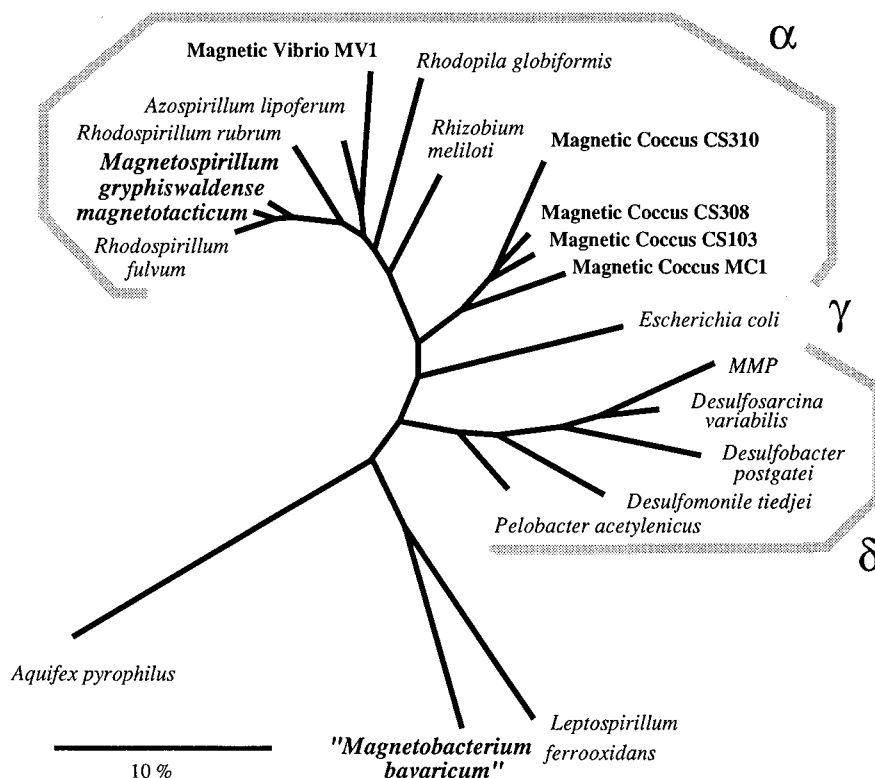


FIG. 7. Phylogenetic tree showing the phylogenetic positions of cultured as well as hitherto uncultured magnetotactic bacteria. The bar indicates 10% estimated sequence divergence.

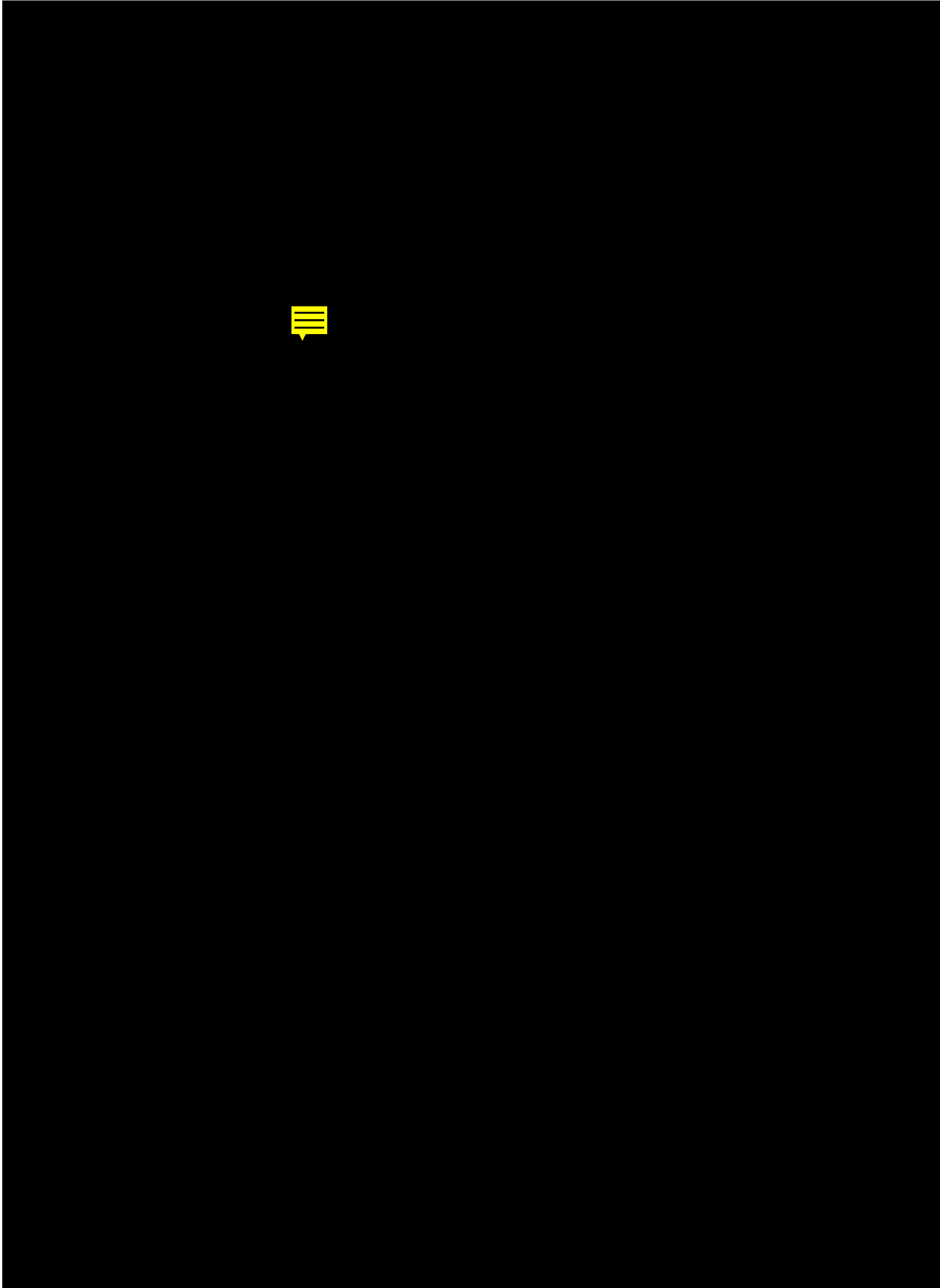
Last but not least, we want to end this long list of symbionts studied by the rRNA approach with the mycoplasmalike bacteria of the order *Mollicutes*, which are characterized by a complete lack of cell walls. On the basis of 16S rRNA cataloging of pure cultures, they have been placed in the gram-positive bacteria with a low GC DNA content (173, 174) (Fig. 5). Members of the *Mollicutes* are difficult to cultivate because of their fastidious nutritional requirements, but over recent years it has become evident that they are widely distributed. Mycoplasmalike organisms are common pathogens of plants, animals, and humans (115, 156); e.g., *Spiroplasma* spp. are found as members of the microbiota in almost all insects and ticks examined (156). Sequencing of PCR-amplified 16S rDNA fragments has facilitated the phylogenetic analyses of hitherto uncultured strains (34, 102, 104, 166).

Magnetotactic Bacteria

Microbial diversity often appears to be overwhelming, as demonstrated by the occurrence of several thousand independent genomes of standard soil bacterium complexity in one soil sample (153). However, considerably less diverse biomass can be obtained by physical separation techniques. One intriguing method is the recovery of magnetotactic bacteria by imposing an artificial magnetic field on mixed environmental samples (see, e.g., reference 97). These bacteria have the ability to orient themselves along the lines of magnetic fields, presumably giving them a higher efficiency in reaching and maintaining optimal positions along chemical gradients. Like other gradient organisms, they are difficult to cultivate. Consequently, "magnetic enrichments" were used for a PCR-assisted retrieval of 16S and 23S rDNA fragments. Comparative sequence analyses of cells retrieved from a sediment sample of a

large freshwater lake (Chiemsee, Upper Bavaria, Germany) still indicated an unexpectedly high diversity, which exceeded the number of observed morphotypes (136) (Fig. 7). Fluorescent-oligonucleotide probing assigned three distinct sequence types to three populations of magnetotactic cocci (CS103, CS308, and CS310) (Fig. 8A). Phylogenetically, these cocci are related to each other and form an independent lineage within the alpha subclass of *Proteobacteria*. Although two cultured magnetotactic bacteria, *Magnetospirillum magnetotacticum* (formerly *Aquaspirillum magnetotacticum*) and *M. gryphiswaldense*, are also alpha-subclass proteobacteria (130), they show no close relationship to the magnetococci (Fig. 7). In situ hybridization demonstrated that the three populations, which were barely discriminated on a morphological basis, had different tactic behaviors (136). More recently, a large magnetotactic rod, unique because of its size (up to 10 μm) and large number of magnetosomes (up to 1,000 per cell), has been identified, phylogenetically analyzed, and monitored in situ by the same approach (Fig. 8A). Microelectrodes were used for a characterization of its microhabitat. The organism, tentatively named "*Magnetobacterium bavaricum*," was found to be a dominant member of a narrow microaerobic zone in the Chiemsee sediment (137).

From sulfidic, brackish-to-marine sediment and water from various coastal sites in New England, cells of a magnetotactic, many-celled prokaryote (MMP) could be collected. Also, three magnetotactic pure cultures could be obtained, including the first coccoid strain to be cultured axenically (MC-1) and two vibrioid strains (MV-1, MV-2). Phylogenetic analyses of 16S rDNAs (31) revealed that MV-1 and MV-2 are identical and form yet another independent line within the alpha subclass. MC-1 is related to the uncultured magnetotactic cocci from the



Chiemsee, and, most surprisingly, the magnetotactic, many-celled prokaryote is a member of the delta subclass of *Proteobacteria* (highest similarity to *Desulfosarcina variabilis*, at 0.91). The authors argued that these findings point to a polyphyletic origin of magnetotactic bacteria and that magnetotaxis based on iron oxides (alpha subclass) and iron sulfides (delta subclass) might have evolved independently. When the 16S rRNA sequence of "*Magnetobacterium bavaricum*" was included in the comparative analyses, this magnetite-containing (108a) magnetotactic bacterium showed a distinct relationship with *Leptospirillum ferrooxidans* (Fig. 7). Both organisms are only moderately related to all other members of the *Bacteria* and presumably form a new phylum. The two possible phylogenetic explanations are that even magnetite-based magnetotaxis evolved independently at least twice or, less likely, that an early ancestor was magnetotactic with magnetite in its magnetosomes. We believe that further studies, applying both molecular and classic isolation techniques, will in the future reveal even more diversity in what was once believed to be a narrow, specialized lineage of bacteria characterized by their intracellular magnetosomes.

Marine Picoplankton

Marine picoplankton samples were the first complex samples studied by direct rDNA retrieval. A considerable amount of effort has created a good collection of partial and more useful full-length 16S rRNA sequences. This reflects the importance of the oceans as the world's largest ecosystems. It is therefore useful to review some of the results.

All studies were performed on DNA extracted from marine picoplankton collected by tangential flow filtration. The preliminary analysis of a subsurface water sample from the Sargasso Sea with domain-specific, rRNA-targeted oligonucleotide probes indicated dominance of members of the domain *Bacteria* in the picoplankton (62). Subsequently, numerous hitherto unknown 16S rRNA sequences were retrieved from the same DNA (60). The analysis of a subsurface water sample from the Pacific Ocean (133) yielded similar results. Interestingly, quite different techniques had been applied for retrieval of rDNA fragments. Schmidt et al. (133) closely followed the approach outlined by Olsen et al. (106); they created a complete genomic DNA library in bacteriophage lambda and identified clones carrying rRNA genes by plaque hybridization with a multiple-kingdom probe. Giovannoni et al. (60), on the other hand, selectively amplified partial 16S rRNA genes by PCR with primers conserved in all bacterial 16S rRNA genes (except for those of the *Planctomycetales*). Although the samples had been taken in different, well-separated oceans and were studied by different techniques, the results of comparative sequence analyses were in good agreement. In both cases, retrieved sequences were related to the 16S rDNA sequences either of cyanobacteria (closest to *Synechococcus* spp. or *Prochloron* spp.) or of the alpha or gamma subclass of *Proteobacteria*. In both samples, closely related alpha-proteobacterial

sequences belonging to a cluster names SAR11 (60) occurred quite frequently. The authors concluded that sequences of this type could be the molecular footprints of a quite abundant but hitherto uncultured group of marine bacteria. By dot blot hybridization with group-specific and universal oligonucleotides, it was determined that rRNA from the SAR11 cluster accounted for 12.5% of the total rRNA in the Sargasso Sea (60). Both groups retrieved additional sequences most closely related to alpha- and gamma-proteobacterial 16S rDNA sequences. None of the retrieved sequences matched an rRNA sequence in the databases; all similarities were lower than 96%. The sequences were clearly distinct from those known in the major genera of cultivated heterotrophic marine bacteria, including *Oceanospirillum*, *Vibrio*, *Photobacterium*, and *Shewanella* (formerly *Alteromonas*). It would be interesting to compare these cloned 16S rDNA sequences with those of the heterotrophic bacteria recently isolated by the microdilution approach (26, 134). Also, it should be stressed that this type of study is very laborious and consequently that only a few dozen clones were examined. When Britschgi and Giovannoni (20) analyzed 51 additional clones retrieved from their Sargasso Sea sample by restriction fragment length polymorphism and hybridization, they identified six distinct groups. These clones included nine clones of the SAR11 cluster, two clones related to marine *Synechococcus* spp., and nine clones forming a second novel alpha-proteobacterial group (SAR83) most closely related (96% similarity) to the aerobic photoheterotroph *Roseobacter denitrificans*. However, the fact that 47% of the examined clones did not fall into any of the recognized groups clearly demonstrated that much more diversity could be retrieved from these rDNA libraries.

When a weak binding of an oligonucleotide probe specific for the domain *Archaea* to DNA isolated from Sargasso Sea samples was observed in 1988, the possibility of cross-hybridization was discussed (62). It came as a big surprise when partial rDNA sequences of archaeal origin could be retrieved from biomass collected at a depth of 10 m in the Sargasso Sea and at depths of 100 and 500 m in the northeastern Pacific Ocean (55, 56). In contrast to earlier studies, PCR was performed with a more universal primer set. Comparative sequence analyses indicated weak affiliation of the new sequences to 16S rDNA of extremely thermophilic archaea. Using a primer set specific for the domain *Archaea*, DeLong (30) retrieved longer (but in the overlapping region, almost identical) sequences from coastal surface water samples from the Atlantic and Pacific. Additional clones indicated the presence of a second group of archaea, related to the methanogens. A probe specific for the domain *Archaea* bound to 4% of the extracted 16S rRNA indicating a significant abundance of this group in the marine plankton (30).

From these studies, it is quite evident that the extent of diversity in marine picoplankton might have been largely underestimated, primarily because of inadequate cultivation techniques. The hitherto cultivated strains represent only a minor part of the true diversity. However, new insights origi-

FIG. 8. (A) Whole-cell hybridization of a magnetotactic enrichment obtained from the microaerobic zone of a freshwater sediment. A phase-contrast (left; magnetosome chains are visible as phase-dense, dark structures) and epifluorescence micrograph (right, double-exposure subsequently with a fluorescein- and rhodamine-specific filter set) allow us to assign two directly retrieved sequences to "*Magnetobacterium bavaricum*" (red rod) (137) and magnetococci of type CS310 (136). Photomicrographs by S. Spring. (B) In situ hybridization of an activated-sludge sample with a probe complementary to a region of the 16S rRNA of most members of the cytophaga-flavobacterium cluster. Various distinct morphotypes, thick and thin rods, a filament, and a large spirillum, are detected by this fluorescein-labeled group-specific probe. Photomicrographs by W. Manz. (C) In situ hybridization of activated sludge with 23S rRNA-targeted oligonucleotide probes specific for the beta (fluorescein labeled) and gamma (rhodamine labeled) subclasses of *Proteobacteria*. Double-exposure epifluorescence photomicrographs clearly show that the original sample (left panel) is dominated by filaments hybridizing with the fluorescein-labeled probe. Addition of a rich broth followed by aerated incubation for 8 h resulted in a complete change in the community composition; the incubated sample (right panel) is dominated by coccoid cells hybridizing with the gamma subclass proteobacterial probe. Photomicrographs by M. Wagner.

nating from the molecular techniques are also still fragmentary. It is therefore necessary not only to extend the sequencing efforts but also to use other approaches to assign the sequences to defined locations, if possible to certain cells. An interesting study in this respect examined the phylogenetic diversity of aggregate-attached (marine snow) versus free-living marine microbial communities (32). Aggregates were drawn into sterile 30-ml syringes, and seawater samples (20 to 40 liters) were prefiltered over a 10- μ m-pore-size Nitex filter at the same depth, time, and site in situ by SCUBA divers. The rRNA genes of the <10- μ m free-living plankton and the aggregate-associated cells were found to be fundamentally different. Whereas most rRNA genes recovered from the prefiltered sample were again related to the alpha-proteobacterial SAR11 cluster, most macroaggregate-associated rRNA clones were related to the cytophaga-flavobacterium cluster, the *Planctomycetales*, or the gamma subclass *Proteobacteria*. Similar techniques could allow greater insight into the puzzling occurrence of sequences related to the anaerobic methanogens or hyperthermophilic crenarchaeota in the cold aerobic oceans. Relating, e.g., the methanogenic sequences to a size fraction characteristic for protozoa would point toward an origin of these sequences in endosymbiotic archaeal cells. A definite morphological or phenotypic characterization of these species will, however, require in situ identification or isolation in pure culture.

Whole-cell hybridization of marine picoplankton samples with fluorescently monolabeled, rRNA-targeted oligonucleotide probes results in signals at and often even below the detection limit of a regular epifluorescence microscope (1). The monitoring of populations on the cell level will therefore require the application of more-sensitive probe assays or detection instruments. It should, however, be feasible to monitor the abundance of specific rRNA or rDNA by quantitative dot blot hybridization of total nucleic acids isolated directly from biomass (113, 146). Given the importance of marine ecosystems, it is surprising that only a few preliminary observations (30, 60, 117) with rRNA-targeted probes have been reported. In one case, strong hybridization of specific probes (based on individual isolates from the same habitat) to community DNA was found in seasonal samples accounting for up to 100% of the hybridization signal obtained with a universal probe (117). This would indicate that the marine picoplankton can, at certain times, be completely dominated by culturable bacteria detected by one specific probe. A second study examined rRNA variations in natural communities of microorganisms on the southeastern U.S. continental shelf (83). Analyses of extracted rRNA revealed that the size fraction corresponding to heterotrophic bacteria responded to the addition of glucose and trace nutrients after a 6-h lag period by a sharp increase in the average rRNA content per cell. A larger (*Synechococcus* spp.) size fraction showed a marked decrease in the average cellular rRNA content during night hours. Both studies used insufficiently characterized probes. There is an obvious need for larger studies applying sets of well-characterized group- and species-specific probes and more-detailed sampling regimes.

Immobilized Microbial Consortia: Biofilms

Planktonic life as individual cells living in aqueous suspensions represents just one possible survival strategy of microorganisms. The second strategy is the colonization of solid surfaces or other interfaces by the formation of so-called biofilms. These immobilized consortia often catalyze important microbial transformations. Likely advantages of this lifestyle are the higher availability of nutrients on surfaces and the possibility of

optimal long-term positioning in relation to other microorganisms or physicochemical gradients (52). Specific analyses of spatial distributions in such systems can only be obtained by techniques like immunofluorescence (for a review, see reference 17) or in situ hybridization. Since the immunofluorescence approach requires the prior isolation of pure cultures for raising specific antibodies and is therefore not really cultivation independent, we will focus here on the use of rRNA-targeted oligonucleotide probes.

Molecular and microscopic identification of defined bacterial populations in multispecies biofilms was first achieved in an anaerobic fixed-bed bioreactor (5). An oligonucleotide complementary to the 16S rRNA molecules of most gram-negative sulfate-reducing bacteria was, together with a conserved primer, used to selectively amplify rDNA fragments. In its fluorescently labeled form, the same oligonucleotide facilitated the detection of resident populations. By in situ hybridization, two morphologically distinct populations (thick and thin vibrios) were visualized and the rapid colonization of newly inserted surfaces could be monitored. Three unique sequences were identified in 12 clones examined. One sequence was closely related (98% similarity in the determined region) to the 16S rRNA sequence of *Desulfovibrio vulgaris*, and another was related to the 16S rRNA sequence of *Desulfuromonas acetoxidans* (96%). The third sequence was specifically but distantly related to spirochete 16S rRNA sequences. This was not totally unexpected, since the specific primer used for amplification has only one or two mismatches to homologous target sites on the 16S rRNA of several other groups. Furthermore, large spirochetes were conspicuous members of the biofilm community. Specific probes were designed on the basis of the retrieved sequences. The *Desulfuromonas*-related sequence could be assigned to thin vibrios and rods, whereas the oligonucleotide targeted to the *D. vulgaris*-related sequence hybridized only to thick vibrios (5). This latter probe was used to monitor enrichments on media optimal for the closest culturable relatives as identified by comparative sequence analysis (77). Although the enrichments were dominated by probe-positive cells, only 3 of 30 isolates were probe positive. All three had identical 16S rRNA sequences 93.9% similar to *D. vulgaris*, and no mismatches were found to the sequence initially amplified from the sulfidogenic bioreactor. The substrate specificity of the one isolate (PT2) selected for further studies was comparable to that of *D. vulgaris*. This nicely illustrates how the rRNA approach can support directed cultivation attempts. By using a combination of fluorescent oligonucleotide probing and quantification of fluorescence intensity by digital microscopy, growth rates of individual cells of the isolate PT2 in the biofilm were estimated (111). This represents an additional important aspect of the rRNA approach, to which we will return below.

The activated-sludge process for the purification of wastewater is, in terms of total metabolized matter, today's most important biotechnological process, yet little is known about the correlations between the community structure and the function of the participating microbial consortia. Activated-sludge flocs can be regarded as mobilized biofilms cometabolizing diverse compounds. Since these consortia are very complex, it was an obvious choice for study by the top-to-bottom approach with oligonucleotide probes specific for different taxonomic levels (160, 161). The first round of hybridizations with oligonucleotides specific for the domains *Bacteria* and *Archaea* (144, 145) have already yielded important results. The majority of cells visualized in activated-sludge flocs by staining with the DNA fluorochrome 4',6-diamidino-2-phenylindole (DAPI) (110) hybridized strongly with a bacterial probe. This indicated

not only in situ dominance of members of the domain *Bacteria* but also high cellular ribosome contents and consequently high growth rates and high metabolic activity of the majority of cells. This was somewhat surprising, since the relatively low ratio between CFU and total cell counts, usually 1 to 15%, had been attributed to a relatively large fraction of dead cells in the recycled biomass. Subsequently, probes specific for the alpha, beta, and gamma subclasses of *Proteobacteria* (92, 160) and other bacterial groups, e.g., the cytophaga-flavobacterium cluster (93) (Fig. 8B), were used to characterize the community structures by in situ hybridization. With each of the group-specific probes, various morphotypes are detected simultaneously, and most (>80%) of the cells hybridizing with a bacterial probe can be classified on this level with a few hybridizations. Comparison of the in situ probing results with probing of the heterotrophic bacteria isolated on nutrient-rich agar plates clearly showed the inadequacy of cultivation-dependent techniques for analyzing the community structure of activated sludge (160). Whereas cells hybridizing with the beta subclass proteobacterial probe were most abundant in situ, most of the colonies could be classified as gamma subclass proteobacteria by probing. Further hybridizations with genus-specific probes revealed that although most (between 30 and 60%) of the colonies obtained represent members of the genus *Acinetobacter*, the in situ abundance of cells hybridizing with the same probe was between only 1 and 10%. Probably, the abundance of *Acinetobacter* spp. in wastewater treatment plants has been overestimated because of their high plating efficiency on the nutrient-rich agars used for enumeration (161). This reinforced the growing appreciation of the selectivity of presumably nonselective media and the inadequacy of most routine culture-dependent methods for describing microbial community structures. The population changes induced by, e.g., addition of nutrients to a microbial community, can be nicely monitored at defined taxonomic levels by probing (160). In Fig. 8C the "gamma shift" occurring in the community structure of an activated sludge sample (untreated original, left panel) within 8 h upon addition of high-nutrient broth (tryptone-soybean broth, 1:1; right panel) was visualized by probing with a rhodamine-labeled probe (conferring red fluorescence) hybridizing to gamma subclass proteobacteria and a fluorescein-labeled probe hybridizing with beta subclass proteobacteria.

An important advantage of the top-to-bottom approach is the fact that as soon as ready-to-use probe sets are developed, they can be applied to a variety of different ecosystems. Hybridization of biofilms developing in drinking-water pipes with the above-mentioned proteobacterial subclass-specific probes revealed that such biofilms are composites of phylogenetically diverse bacteria (95). Another example is the monitoring of growth of pathogenic *E. coli* strains in biofilms formed by a gram-positive, benzoate-degrading bacterium by in situ hybridization with the gamma subclass proteobacterial probe. In biofilms maintained on a medium containing 0.5 mM sodium benzoate as the sole carbon source, target cells grew to high densities, even though the studied *E. coli* strain could not utilize benzoate (152). After several weeks, *E. coli* cells started to be released in large numbers into the effluent, an observation questioning the value of this organism as a fecal indicator for water quality control. In both experiments bright fluorescence signals were indicative of relatively high cellular ribosome contents and consequently of high physiological activity of the target cells. As a general observation, in one and the same water body, microbial cells attached to surfaces are more readily detectable with rRNA-targeted probes than are planktonic cells (95).

In addition, the top-to-bottom approach has the advantage

that bookkeeping between the hybridization signals obtained with probes at the different levels can indicate hitherto undetected diversity. This was nicely explained for quantitative dot blot hybridization by Devereux et al. (35): "If all existing (known) subgroups have been targeted, then amounts of rRNA hybridized by specific probes should total the amounts hybridized by a general probe. A greater amount of rRNA determined by hybridization with a more general, group-specific probe would indicate the presence of an additional (hitherto unknown) rRNA subgroup(s) that had not been accounted for by the specific probes."

The top-to-bottom approach is far less time-consuming than an analysis of rDNA libraries by sequencing. The abundance of defined populations can be determined much more accurately. However, this approach cannot, of course, substitute for the direct retrieval of sequences. When applied together, probing and sequencing are synergistic. Comparative probing of the original sample, the extracted nucleic acid pool, and the corresponding rDNA library with phylogenetically nested group-specific oligonucleotides is the best way to evaluate the representativeness of the retrieved rDNA sequences and to identify the procedure that causes the biases.

Detection of Soil Bacteria

Soils represent probably the most complex and the most difficult of environments to study. As mentioned above, there is good evidence there might be several thousand microbial species in one soil sample (153). Also, microbiologists applying the rRNA approach to soil samples still find it very challenging. Because of the high complexity, little information of ecological relevance can be obtained by pure-sequence retrieval. Not unexpectedly, a clone library generated from DNA directly extracted from a subtropical Australian soil sample contained substantial diversity (88, 141). By comparative sequence analysis of 30 clones and dot blot hybridization of 83 additional clones with defined oligonucleotide probes (88), three major groups were found: (i) 57 clones were related to the 16S rRNA of nitrogen-fixing bacteria of the alpha-2 group of *Proteobacteria*; (ii) 7 clones were affiliated with the order *Planctomycetales* without close similarity to any described *Planctomyces* spp.; and (iii) 22 clones fell in a new line of descent, sharing a common ancestry with the planctomycetes and chlamydiae. Of the additional 25 clones that could not be grouped by hybridization, 5 were sequenced but could not be convincingly affiliated to known groups by comparison with available rRNA data bases. Whereas the retrieval of sequences related to the alpha subclass of *Proteobacteria* was not surprising, the planctomycete-related sequences came as a surprise, since planctomycetes had previously been detected only in aqueous habitats. More recently, phylogenetic analysis of 20 additional clones revealed the presence of three novel clusters (141). One of these clusters is affiliated with the alpha-1 subdivision of *Proteobacteria*, and two form hitherto unknown lineages within the gram-positive bacteria. No probing data were presented to quantify the abundance of cells or specific rRNAs in the original sample. Considering that PCR was part of the generation of the clone library, it can only be speculated that these sequences originated in the autochthonous microbiota of the examined soil. However, this study again shows how much more diversity is awaiting discovery. Some of the obstacles observed when analyzing such complex samples will be discussed in detail in the following section.

CURRENT OBSTACLES TO GENERAL APPLICATION OF THE rRNA APPROACH

Difficulties in the application of the rRNA approach can occur in both sequence retrieval and probing of highly diverse samples.

Difficulties in Sequence Retrieval

Formation of chimeric rDNA sequences. A potential risk of gene amplification by PCR from mixed-culture DNA is the formation of chimeric sequences assembled from sequences of different species. That this is more than a theoretical threat was demonstrated by Liesack et al. (89). Sequence analysis of five clones obtained from an apparently pure culture of a strictly barophilic, psychrophilic bacterium revealed the presence of two different, moderately related (90% similarity) 16S rDNA types (A and B). A sixth clone contained a chimeric rDNA insert which was approximately 1,000 nucleotides identical to type A at the 5' end and approximately 500 nucleotides identical to type B at the 3' end. Two main factors will increase the probability of chimera formation: (i) the availability of partial-length rDNA fragments present in low-molecular-weight genome DNA preparations or generated by premature termination of elongation during PCR and (ii) the percentage of highly conserved stretches along the primary structure of rDNA types, where, after denaturation, single strands originating from different rDNAs can anneal in highly complementary regions. The lengths of these regions are expanded with an increasing degree of relationship between the organisms investigated. If one partial-length rDNA fragment of organism A binds to a full- or partial-length rDNA fragment of organism B one or two full-length chimeric sequences, respectively, will be generated in the next elongation step. Further amplification of the chimeric sequences proceeds with the same efficiency as for nonchimeric fragments. Starting from high-molecular-weight DNA, chimeric clones are found only at a frequency of several percent in rDNA libraries obtained from complex environmental samples (e.g., 2 in 113 analyzed clones [88]). This artifact may incorrectly be interpreted as an indication of additional biodiversity in the natural sample. Whereas real diversity is represented by two distinct populations with two different rDNA types, the clone library might indicate the presence of a continuum of rDNA types with only small differences between the individual types. These differences would be within the limits defined by the two actual rRNA types. It is obviously important to sort out such chimeric sequences. Several PCRs should be performed, and multiple clones should be sequenced. A chimera can then be identified either by checking the complementarity of helical regions or by performing comparative sequence analyses of different sections of the rDNA amplification products (89). Phylogenetic trees reconstructed for different sections, e.g., the 5' and 3' halves, of a chimeric clone would indicate different affiliations. The rRNA database project at the University of Illinois supplies a chimera check program (86).

Fishing for rDNA sequences of less common organisms. From a given gene library, the number of clones analyzed has usually been limited to several dozen. With modern sequencing techniques combined with approaches for detection of already analyzed clones by oligonucleotide probing (see, e.g., references 20 and 88) or restriction fragment length polymorphism (20, 32), this number might be increased to several hundred or thousands. However, it is a clear methodological limit of the standard rRNA approach that less common organisms (e.g., abundance below 1 in 1,000) will hardly be detected. For morphologically conspicuous, nonculturable microorgan-

isms, a straightforward way out of this dilemma is micromanipulation. This approach has been taken to study the phylogeny of extremely large microorganisms (*Epulopiscium fishelsoni*) inhabiting the intestinal tracts of surgeonfish (8), where individual cells could be collected from ethanol-fixed gut contents.

Alternative techniques are available. Modern flow cytometers allow automated cell sorting. In a recent study, the characteristic forward and large-angle light scattering of large magnetotactic bacteria was used to further enrich this morphotype from a cell suspension obtained with a bar magnet (137). This was necessary since, for unknown reasons, rDNA clones of this abundant bacterium were underrepresented after amplification and cloning. The applicability of optical trapping with infrared lasers for micromanipulation of individual bacterial cells was demonstrated several years ago (10). Today, several groups are trying to combine this fascinating technique with the rRNA approach.

Retrieval of a less common sequence can also be facilitated by the use of one or two specific primers for PCR amplification. If probing with a group-specific oligonucleotide suggests a low abundance of a certain population, its rDNA can be selectively amplified by using the same oligonucleotide(s) as a primer(s) for PCR (5). Furthermore, with techniques like denaturant gradient gel electrophoresis, it is possible to physically separate PCR products originating from the amplification of rDNA fragments from environmental samples. Besides obtaining a characteristic fingerprint for the examined community, the possibility of isolating less common fragments should allow their specific retrieval (101).

Problems with In Situ Hybridization

No cells, no signals. The first point in troubleshooting can be summarized in a simple line: "no cells, no signals." Whole-cell hybridization can identify an individual cell; however, it is usually not trivial to bring this cell into the examined microscopic field(s). At a magnification of $\times 1,000$, an average microscopic field has a diameter of about 200 μm . Let us assume that 10 μl of a cell suspension is spotted in a circle with a diameter of 1 cm and that all cells are immobilized by air drying. In this example, the microscope factor, which is defined as the area containing cells divided by the area of one microscopic field, is 2,500. A 10- μl sample with a cell concentration of 10^7 cells per ml brings 10^5 cells into the examination area, which corresponds—considering the microscope factor—to only 40 cells per average microscopic field. Accordingly, 10 μl of a cell suspension with a concentration of 10^5 cells per ml introduces 10^3 cells into the examination area, so that on the average, 2.5 fields have to be viewed in order to detect one cell. Obviously, it becomes very tedious to detect less than 10^3 cells per cm^2 . Keeping this in mind, it is quite obvious that target cells often have to be concentrated. This can be quite easy, e.g., for aquatic samples that can be filtered, or quite problematic, e.g., for sediment or soil samples in which noncellular particles have to be removed prior to concentration. If cells from a 10-liter water sample can be concentrated by a combination of tangential-flow filtration and membrane filtration within an area of 1 cm^2 , even concentrations as low as 10 cells/ml will result in easily analyzed cell densities ($10^5/\text{cm}^2$).

Furthermore, it should be pointed out that although it is possible to detect individual cells in the presence of 10^6 non-target cells (1), technical constraints probably prevent reliable detection of, e.g., one beer spoilage bacterium against a background of 10^8 yeast cells.

Low signal intensity. Low signal intensity after in situ hybridization is another frequently encountered problem. If

nothing is wrong with the probes, this problem can be caused only by small numbers or insufficient accessibility of the target molecules, in our case the rRNA molecules.

Correlation between growth rate and cellular rRNA content.

A direct correlation between rRNA content and growth rate of bacteria was first shown by Schaechter et al. in 1958 for *Salmonella typhimurium* (127). Accordingly, the signals conferred by fluorescently labeled rRNA-targeted probes to whole fixed *E. coli* cells were correlated to cellular rRNA contents and growth rates (33, 162). This has two consequences: (i) slowly growing cells are difficult to detect because of their low cellular rRNA content, and (ii) the quantification of probe-conferred fluorescences should allow estimation of in situ growth rates of individual cells. The former, more negative aspect was reflected in an analysis of fixed cells of *Burkholderia (Pseudomonas) cepacia* and *E. coli* with monolabeled oligonucleotides. Here, with increasing generation times, the hybridization signals were quickly approaching the detection limit of a regular flow cytometer, which is around a few thousand fluorochromes (162). The positive aspect of this correlation—namely, that rRNA-targeted probes allow more than just identification—was recently exploited for the first time. Probing with a specific fluorescent oligonucleotide probe was combined with digital microscopy, using a cooled charge-coupled device camera to quantify the fluorescence emitted from individual cells of a bacterial sulfate reducer growing in a multispecies biofilm (111). By correlating the determined intensities with those obtained from cell suspensions of the same strain growing at different rates, the in situ generation times were found to be around 35 h in a young biofilm. In established biofilms, the doubling time was considerably longer. One should, however, be cautious in considering this more than an estimate. It is unclear whether correlations between growth rates and rRNA content determined for cell suspensions also hold for attached growth in biofilms. Furthermore, there is good evidence that the classic correlation does not apply to carbon-starved cells. Cells of *Vibrio* sp. strain S14 that have been deprived of exogenous carbon for several days and were not growing under these conditions retained quite large numbers of ribosomes (51). The protein synthesis capacity far exceeded the apparent demand for translation and may be essential for the ability of this *Vibrio* strain to immediately regain high activity as soon as starvation is terminated by addition of a substrate. It remains to be examined if this finding for a recently isolated environmental strain can be generalized. Quite frequently, new isolates behave differently from established laboratory strains. It is also necessary to perform more basic research on slowly growing bacteria before probing with rRNA-targeted hybridization probes for the in situ determination of growth rates. Such studies have been performed on the marine denitrifying bacterium *Pseudomonas stutzeri* Zobell (79) and four marine bacterial isolates of which three were members of the gamma subclass of *Proteobacteria* (78). Generally, the RNA content decreased with decreasing growth rate. Different measures of RNA content (assayed by ethidium fluorescence) such as RNA per cell, RNA/biovolume ratio, RNA/DNA ratio, and RNA/DNA/biovolume ratio were significantly different among isolates (78). Interestingly, the correlation between the total RNA/DNA ratio of *P. stutzeri* and the growth rate was linear for generation times between 6 and 60 h, which are realistic for marine bacteria. Kerkhof and Ward (79) also used a 200-bp nucleic acid probe derived from the 23S rRNA of *P. stutzeri* Zobell to determine rRNA/rDNA ratios. A significant but not simply linear decrease of this ratio with decreasing growth rate was found. Such rRNA/rDNA hybridizations with species-specific probes could allow one to assay for specific growth rates in

mixed culture and ultimately in the marine environment (79). Unfortunately, four of the five strains examined in these two studies are members of the gamma subclass of *Proteobacteria* and the fifth could not be placed as a result of incomplete sequence data. Future studies are needed to show if the described correlations also apply to members of other important phylogenetic lineages, such as the alpha subclass of *Proteobacteria* or the cyanobacteria.

Target accessibility. When a rapidly growing culture with presumably high cellular rRNA content exhibits low signal intensities, it has proven useful to distinguish between cases in which cell peripheries limit diffusion of rRNA-targeted probes into whole fixed cells and cases in which higher-order structures in the ribosomes—both RNA-RNA and RNA-protein—prevent probe hybridization. The former will hinder probe binding independently of the exact location on the rRNA target site. The phenomenon is best understood as an exclusion of a certain molecule size, probably by the cell wall, since membranes are expected to be readily permeable after fixation. Such cell wall-limited probe accessibilities were encountered with aldehyde-fixed gram-positive bacteria (67, 126, 129). Treatment of paraformaldehyde-fixed cells with cell wall-lytic enzymes like lysozyme increased the permeability of gram-positive lactococci, enterococci, and streptococci (14) and *Streptomyces scabies* hyphae (67). Obviously, enzymatic treatments will permeabilize only sensitive cell walls. It is difficult to achieve a good compromise between sufficient cell permeabilization for efficient hybridization and good preservation of morphological detail. Fixatives fall into two general classes, (i) precipitants like ethanol or methanol and (ii) cross-linking agents like the aldehydes (66). Whereas a 3% paraformaldehyde solution is a good fixative for most gram-negative bacteria, it was shown to be detrimental for whole-cell hybridization of gram-positive bacteria with fluorescently labeled oligonucleotide probes (18). The same gram-positive cells could, however, be detected after fixation by heat (76) or with ethanol-formalin (9:1, vol/vol) (18) or 50% ethanol (122). This illustrates that fixation procedures should be considered an integral part of in situ hybridizations.

Less predictable is the in situ accessibility of defined target sites on either 16S or 23S rRNA molecules. We are routinely evaluating whole fixed cells with probes that have proven to result in bright hybridization signals with a wide array of microorganisms, e.g., the probes UNIV1392 (146) or EUB338 (145) that are complementary to regions of the 16S rRNA molecules of all sequenced microorganisms or all bacteria, respectively. Strong fluorescence of a cell preparation is taken as an indication for both a sufficient cellular rRNA content and a good permeabilization of the cell wall. Nevertheless, newly designed probes may fail to give similarly bright fluorescence signals with the same cells. First, factors like noncomplementarity of probe and target, ineffective probe labeling, or non-optimal hybridization conditions (e.g., hybridization at a temperature above the temperature of dissociation [T_d] of the probe target hybrid) must be avoided. After an error on this level has been ruled out, for example by successful hybridization of the same probe against isolated rRNA, everything points toward an in situ inaccessibility of the probe target site. Since the rRNA target molecules remain in the ribosomes of the whole fixed cells, probe hybridization is much more influenced by RNA-RNA or RNA-protein interactions. Although the higher-order structure of the ribosome is well known, the configurational effects of fixation can hardly be predicted. In situ accessibility can sometimes be improved by addition of formamide to the hybridization buffer (5). Addition of this denaturing solvent weakens the effects of hydrogen bonds,

thereby "softening" hinderance by higher-order structures. The in situ accessibility of rRNA molecules for oligonucleotide probes has hitherto not been examined in a systematic way. There is some indication (although from negative results) that certain regions might be inaccessible in certain phylogenetic groups. Sometimes, shifting the target site by just a few nucleotides has a major influence on the probe sensitivity (1). A good hint for suitable in situ probing sites can be retrieved from a listing of successful whole-cell hybridization probes (Table 3). In Fig. 9, the information on the target sites is presented on secondary-structure models of bacterial 16S and 23S rRNAs.

INCREASING THE SENSITIVITY OF WHOLE-CELL HYBRIDIZATION

Currently, the standard probes are rRNA-targeted oligonucleotides with a single fluorescent dye molecule attached to the 5' end via a linker molecule. The most frequently used dyes are fluorescein (excitation wavelength, 490 nm; emission wavelength, 520 nm), tetramethylrhodamine (550 nm, 575 nm) and Texas Red (578 nm, 600 nm). Alternative dyes, e.g. 7-amino-4-methylcoumarin-3-acetic acid (80) and the indocarbocyanine dyes CY3, CY5, and CY7 (135), are becoming readily available. The dye sensitivity is essentially determined by the product of quantum yield and molar absorbance, but its in situ detectability is also influenced by nonspecific binding and the autofluorescence of cells and surrounding material. As outlined above, fluorescently monolabeled probes might fail to detect cells with small numbers of ribosomes. Consequently, with notable exceptions like the detection of cells in biofilms formed in drinking-water pipes (95), most successful applications have been restricted to eutrophic environments (e.g., activated sludge [159-161]). Detection rates were low in picoplankton samples from oligotrophic waters (1) or soils (67).

Probes can be made more sensitive by one or a combination of the following approaches: (i) indirect labeling; (ii) the use of alternative, more sensitive labels; or (iii) multiple labeling. Depending on the cell type and the environment, one particular approach will be more sensitive than another.

Indirect Assays

The principle behind indirect labeling is the attachment of a reporter molecule to the nucleic acid probe, which, following hybridization, is detected in a second step by a labeled binding protein. For biotin, this binding protein is either avidin or streptavidin. For the steroid digoxigenin and other reporters, detection is achieved with specific antibodies. It has been demonstrated that 5' and/or 3' digoxigenin-labeled oligonucleotide probes facilitate whole-cell detection both with fluorescently and enzymatically labeled antibody fragments (178). When fluorescent-antibody fragments are used, the potential increase in sensitivity compared with the standard directly fluorescein-monolabeled oligonucleotides is limited by the molar fluorescent dye/protein ratio, which is around 3. However, combined with enzyme-labeled antibodies and the precipitation of suitable substrates, this approach allows detection of individual cells even against a strongly autofluorescent background, e.g., the detection of symbiotic bacteria in the ovarian tissues of clams (27) or on the cuticula of nematodes (109). Applications of biotin-labeled, rRNA-targeted oligonucleotides include the detection of *Pneumocystis carinii* in lung sections with alkaline phosphatase-labeled streptavidin and the detection of marine nanoplanktonic protists with fluorescein-labeled avidin (90). A related technique is the detection of sulfonated oligonucleo-

tides by anti-sulfonated DNA antibodies; this was used for the in situ detection of mycobacteria in biopsy specimens (9). It should be considered that all indirect approaches rely on the free penetration of the labeled binding protein to the probe-conferred reporter molecules. Since these molecules are considerably larger than fluorescently labeled oligonucleotides, the fixed target cells may have to be further permeabilized by enzymatic or chemical treatments (9, 41, 178).

Enzyme-Labeled Oligonucleotides

It is also possible to detect whole fixed cells with oligonucleotides that are covalently linked to enzymes like horseradish peroxidase (6). Again, probe-conferred enzyme is detected via the formation of an insoluble colored precipitate from suitable substrates. This technique is most helpful when a strong background fluorescence or cellular autofluorescence prevents or disturbs the detection of probe-conferred fluorescence, e.g., the detection of symbiotic cyanobacteria in plant sections (177a) (Fig. 10A). Compared with the indirect approaches, this technique is faster and less prone to specificity problems, since the antibody/avidin step is avoided. In terms of size, oligonucleotide-horseradish peroxidase conjugates are between the smaller fluorescent oligonucleotides and the larger antibody-enzyme complexes. Recent experiments indicate that cell permeabilization can be achieved for most (all hitherto examined) gram-negative and several gram-positive bacteria by fixation with ethanol. It still has to be shown that directly and indirectly enzyme-linked hybridizations are superior to the standard fluorescence hybridization assays for the detection of microbial cells with low cellular rRNA contents. The suitability of other labels for whole-cell probing of rRNA should be further examined to develop an arsenal of assays compatible with different environmental samples and microscopic techniques, e.g., gold-labeled probes for electron-microscopic studies.

Multiple Labeling

There have been several attempts to increase the sensitivity of whole-cell hybridization by directing multiple fluorescent dye molecules to one ribosome. By using several monolabeled oligonucleotides to different target sites on the 16S and or 23S rRNA molecules, more fluorescence is directed to the target cells (1, 87). Although the fraction of detected cells might be significantly increased by this approach (e.g., from 20% with one probe to 75% with five probes [87]), the general applicability of this approach is restricted by the relatively limited availability of target sites with identical specificity. For unknown reasons, experiments with oligonucleotides carrying several dye molecules within the hybridizing parts or in non-hybridizing tails did not result in improved signals (162). Recently, 23S rRNA-targeted polyribonucleotide probes (up to 260 nucleotides in length) were produced by in vitro transcription and labeled with UTP derivatives (fluorescein-12-UTP, 7-amino-4-methylcoumarin-3-acetyl-6-UTP, tetramethylrhodamine-6-UTP, or digoxigenin-11-UTP). Despite their length, these molecules hybridized to whole, fixed, gram-negative cells (154). Fluorescence intensities were quantified for target and nontarget cells with a combination of a charge-coupled device video camera and an image-processing system. Polyribonucleotide probes conferred up to 26 times more fluorescence to target cells than oligonucleotide probes did. Probe sensitivity and specificity were strongly influenced by the stringency of hybridization. Maximal sensitivity was achieved by applying relatively relaxed hybridization conditions which resulted in the detection of several species within one genus. Under conditions sufficiently stringent for detection of one species, the

TABLE 3. Sequences, target sites (*E. coli* numbering [22]), and specificities of rRNA-targeted oligonucleotide probes used for whole-cell hybridization^a

Sequence (5'-3') of probe	Target position	Specificity	Reference(s)
Universal			
ACGGGCGGTGTGTRC	16S, 1392-1406	Universal	106, 146
GWATTACCGCGGCKGCTG	16S, 522-536	Universal	61
CGACGTTYTAAACCCAGCTC	23S, 2576-2595	Universal	90a
Domain level			
GTGCTCCCCGCCAATTCCT	16S, 915-934	Archaea	144
TCCGGCRGGATCAACCGGAA	16S, 2-21	Archaea	61
TTGYAGC(C/I)CGCGTGY(M/I)GCCCSGS(M/I) S(A/I)TCGGGGC	16S, 1206-1239	Archaea	33
GCTGCCTCCCGTAGGAGT	16S, 338-355	Bacteria	2, 145
ACCGCTTGTCGGGGCCC	16S, 927-942	Bacteria	61
TGAGCCAGGATCAAACTCT	16S, 8-26	Bacteria	72
CTACCAGGGTATCTAATCC	16S, 785-803	Bacteria	87, 171
CACGAGCTGACGACAGCCAT	16S, 1055-1074	Bacteria	87, 171
GCTCGTTGCGGGACTTAACC	16S, 1088-1107	Bacteria	87, 171
ACCCGACAAGGAATTTCCG	23S, 1933-1951	Bacteria	90a
ACCAGACTTGCCCTCC	16S, 502-516	Eucarya	1
GGGCATCACAGACCTG	16S, 1195-1209	Eucarya	61
TAGAAAGGGCAGGGA	16S, 1379-1394	Eucarya	72
Upper group level			
CGTTCGYTCTGAGCCAG	16S, 19-35	Alpha subclass of <i>Proteobacteria</i> , several members of delta subclass of <i>Proteobacteria</i> , most spirochetes	92
GCCTTCCCACTTCGTTT	23S, 1027-1043	Beta subclass of <i>Proteobacteria</i>	92
GCCTTCCACATCGTTT	23S, 1027-1043	Gamma subclass of <i>Proteobacteria</i>	92
CGGCGTCGCTGCGTCAGG	16S, 385-402	Most members of delta subclass of <i>Proteobacteria</i> , few gram-positive bacteria	1
TGGTCCGTGTCTCAGTAC	16S, 319-336	Cytophaga-flavobacterium cluster of CFB phylum	93
CCAATGTGGGGGACCTT	16S, 303-319	Bacteroides cluster of CFB-phylum	93
TATAGTTACCACCGCCGT	23S, 1901-1918	Gram-positive with high-GC DNA content	122
CTTGCCCRGCCCTT	16S, 498-510	Most euryarchaeota	25
CCAGRCTTGCCCCCGCT	16S, 499-515	Most crenarchaeota	25
Lower group level/genus level			
CCCTGAGTTATCCGAAC	16S, 142-159	Methylotrophic bacteria	155
GGTCCGAAGATCCCCCGCTT	16S, 197-216	Methylotrophic bacteria	155
TAACCCAGGGCGGACGAG	23S, 1325-1342	<i>Lactococcus lactis</i> and relatives	90a
GCTGGCCTAGCCTTC	23S, 1432-1446	Most true <i>Pseudomonas</i> spp.	128
GCTGGCCTAACCTTC	23S, 1432-1446	<i>Pseudomonas putida</i> and <i>P. mendocina</i>	128
TTCCACATACCTCTCTCA	16S, 659-676	<i>Brevundimonas diminuta</i> and several <i>Caulobacter</i> spp.	128
TTCCATCCCCCTCTGCCG	16S, 659-676	<i>Comamonas testosteroni</i> , <i>Leptothrix discophora</i> and relatives	128
GCAGTTACTCTAGAAGACGTTCTTCCCTGG	16S, 442-470	<i>Bacillus polymyxa</i> and <i>B. macerans</i>	76
CTCTGCCGCACTCCAGCT	16S, 649-666	<i>Leptothrix discophora</i> , <i>Aquaspirillum metamorphum</i>	159
CATCCCCCTCTACCGTAC	16S, 656-673	<i>Sphaerotilus natans</i> and relatives	159
TTCCACTTTCCTCTACCG	16S, 659-676	<i>Holospora</i> spp.	4
ACTACCCTCTCCGTGATT	16S, 655-672	<i>Magnetospirillum</i> spp.	130
TMCGCARACTATCCCCAAA	16S, 220-239	<i>Desulfobacter</i> spp.	1
ATCCTCTCCATACTCTA	16S, 652-669	<i>Acinetobacter</i> spp.	161
CTGTGTTCCCTTCCGATC	16S, 705-722	Most <i>Legionella</i> spp.	94
Species level			
CATCCTGCACCGCAAAAAGCTT	16S, 196-230	<i>Mycobacterium leprae</i>	9
CCTCTCGATCTCTCTCAAGT	16S, 1006-1024	<i>Synergistes jonesii</i>	96
ACCATCCTCTACCGAACT	16S, 655-672	Magnetotactic coccus CS308	136
ACCACCCTCTGCCAACT	16S, 655-672	Magnetotactic coccus CS310	136
ACCACCCTTGCCGGACT	16S, 655-672	Magnetotactic coccus CS103	136
GCCATCCCCTCGTTACT	16S, 655-672	" <i>Magnetobacterium bavaricum</i> "	137
GGACTACCCCTTAAACGG	16S, 218-235	Magnetotactic bacterium (MMP)	31
TCTCCGAACTCAAGTCCA	16S, 647-665	Sulfate-reducing bacterium (PT2)	5
TCTCCGTAATCAAGTCTG	16S, 647-665	Sulfate-reducing bacterium (PT1)	5
AACACCCTACTCTTTTCG	23S, 1428-1447	<i>Holospora obtusa</i>	4
TTCCACTTTCCTCTCTCG	16S, 659-676	<i>Caedibacter caryophila</i>	139
ACTACCCTCTCCATACT	16S, 655-672	<i>Sarcobium lyticum</i>	140
GCTGTACTCAAGTTACCCAGTTCTAA	16S, 637-662	Bacterial endosymbiont	37
CCCGTAAGSCCGACACCTAGTATT	16S, 822-846	<i>Epulopiscium fishelsoni</i>	8
TTGCGGTTAGGTCACGTGACGTTGGGCCCCCT	16S, 1423-1453	<i>Epulopiscium fishelsoni</i>	8

Continued on following page

TABLE 3—Continued

Sequence (5'-3') of probe	Target position	Specificity	Reference(s)
TTCCACCCCTCTACCA	16S, 659-676	Bacterial ectosymbiont	109
TTTCCCCCTCAGGGCGT	16S, 181-197	<i>Polynucleobacter necessarius</i>	138
CCGGTCTCGAGCCTAGCA	16S, 644-661	Archaeal endosymbiont	138
TCCCGACCTCAAGTCTAA	16S, 646-663	Archaeal endosymbiont	138
CAGGATACATCCACTATG	16S, 178-194	Archaeal endosymbiont	43
CTGCATCGACAGGCACT	16S, 403-429	Archaeal endosymbiont	43
AACCCGTACAGATCAAAGG	16S, 276-294	Archaeal endosymbiont	44
GACCATTCCAGGAATCTCTA	16S, 184-202	Archaeal endosymbiont	44
TTTACGTTTGCCCATTC	16S, 497-498*	<i>Metopus palaeformis</i>	44
TTCGGAGGACCAGGCAACCAATCCCT	16S, 651-652*	<i>Pneumocystis carinii</i>	41
AAGGAAAATGAACTTGCTGGCTCTG	16S, 1269-1270*	<i>Pneumocystis carinii</i>	41
CTTTCCAGTAATAGGCTTATCG	16S, 1453-1461*	<i>Pneumocystis carinii</i>	41
GTTGAAGCTTGACGGTATATCGCAAACCTCCTA	16S, 827-859	<i>Porphyromonas gingivalis</i>	59
GTAGAGCTTACTATATCGCAAACCTCCTA	16S, 827-859	<i>Bacteroides forsythus</i>	58
CCCATCGCATCTAACAAT	23S, 1406-1423	<i>Burkholderia cepacia</i>	128
TAAAGGACCCACCATTCTCT	16S, 1020-1042	<i>Frankia</i> sp.	69
GCAGGACCTTACGGATCCC	16S, 1020-1042	<i>Frankia</i> sp.	69
TCCCATGCAGGAAAAGGATGTATCGGGTAT	16S, 172-193	<i>Bacillus polymyxa</i>	76
CTCCATATCACTACTTCGCTTCCCGTTGTA	16S, 1235-1269	<i>Bacillus macerans</i>	76
GCCTACCTCAACCTGATT	16S, 655-672	<i>Haliscomenobacter hydrossis</i>	159
CCCCTCTCCAAACTGTA	16S, 652-669	<i>Leucothrix mucor</i>	159
CTCCTCTCCACATTCTA	16S, 652-669	<i>Thiothrix nivea</i>	159
TCCCTCTCCAAATTCTA	16S, 652-669	Filamentous bacterium 021N	159
TGCCCTGAACCTATCCAAGA	16S, 650-669	<i>Fibrobacter succinogenes</i>	2
CCGCATCGATGAATCTTTCGT	16S, 207-226	<i>Fibrobacter intestinalis</i>	2, 146
GCCCCGTGCCATTGTACGCC	16S, 1242-1265	<i>Fibrobacter intestinalis</i>	2
CCGGTCTTCTTCTGTAGGTAACGTCACAG	16S, 477-506	<i>Shewanella putrefaciens</i>	36
CTATAATGCTTAAGTCCACG	23S, 271-289	<i>Lactococcus lactis</i>	90a
CATACCTTCGCTATTGCT	23S, 1866-1883	<i>Streptococcus salivarius</i>	14, 42
CATGCCTTCGCTTACGCT	23S, 1866-1883	<i>Streptococcus thermophilus</i>	14, 42
GGTGTGTTAGCATTTCCG	23S, 343-360	<i>Enterococcus faecalis</i>	14, 16
CACACAATCGTAACATCC	23S, 142-158	<i>Enterococcus faecium</i>	14, 16
GATAGTCACTAGGTTTCG	23S, 673-691	<i>Lactococcus lactis</i>	90a
ACCGTTCGTCTAACACAT	23S, 287-304	<i>Vibrio vulnificus</i>	11
Subspecies level			
TGCAAGCACCAATCTTCATC	16S, 67-88	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	14, 126
ATTAATAATAATCCAAAGCTT	23S, 1151-1180	<i>Lactococcus lactis</i> subsp. <i>lactis</i> DSM 20481	14, 16
CCATACCGATAAATCTCTAGT	16S, 207-226	<i>Fibrobacter succinogenes</i> subsp. <i>succinogenes</i>	2, 146

* *, target site on an insertion not present in *E. coli*; I, inosine; M = A or C; R = A or G; K = G or T; S = G or C; Y = C or T; W = A or T.

increase in probe-conferred signal compared with monolabeled oligonucleotide probes was only marginal. Nevertheless, the increased sensitivity of the multiple labeled polynucleotides facilitates, e.g., the use of an additional, less bright, blue fluorescent dye (aminomethylcoumarine) for whole-cell hybridization and thereby the monitoring of a third population (besides the ones detected by green and red fluorescent probes) in one sample (Fig. 10B).

Instrumentation

Obviously, the overall sensitivity of whole-cell hybridization will also depend on the instrumentation used for detection. There are several possibilities to increase the detectability of probe-conferred fluorescence signals. In samples with low background, highly sensitive charge-coupled device cameras can visualize low levels of emitted light. In samples with broad spectra of intrinsic fluorescence, optimized narrow-band-pass filter sets and analysis of digitized images may facilitate the detection of cells (112). One of the many possible procedures includes the digitization of one microscopic field with two different filter sets. A black-and-white image of the intrinsic green fluorescence as determined, e.g., by excitation with blue light, is subtracted pixel by pixel from the black-and-white image obtained with a rhodamine-specific filter set. This re-

duces the noise caused by the broad inherent fluorescence and facilitates more clear-cut imaging of the probe-conferred fluorescence.

Another piece of equipment that can solve many of the problems encountered during fluorescent in situ probing of microbial populations is the laser confocal scanning microscope. The technique of optical sectioning removes out-of-focus fluorescence and leads to sharp and clear images. Especially in cases when cells are immobilized in or on fluorescent materials, e.g. azospirilla on the surface of wheat roots (132), conventional fluorescence microscopy fails to detect stained cells, since fluorescence is collected from the total depth of the sample. By restricting the collected signal to a thin section (e.g., 1 μm), most of the noise is removed and bacteria are visualized much more clearly. The laser confocal microscope is an ideal tool for analysis of spatial distributions, e.g., of immobilized microbial communities like those forming the activated-sludge flocs. Red-green three-dimensional images have been reconstructed from the data obtained by serial optical sectioning (Fig. 10C; for a three-dimensional impression, red-green three-dimensional reconstructions have to be viewed with appropriate red-green glasses) of a fixed activated-sludge sample hybridized with the beta (left panel) and gamma proteobacterial probes. In Fig. 11, the data of the left red-green three-dimensional image are presented as a series of 16 xy sections at

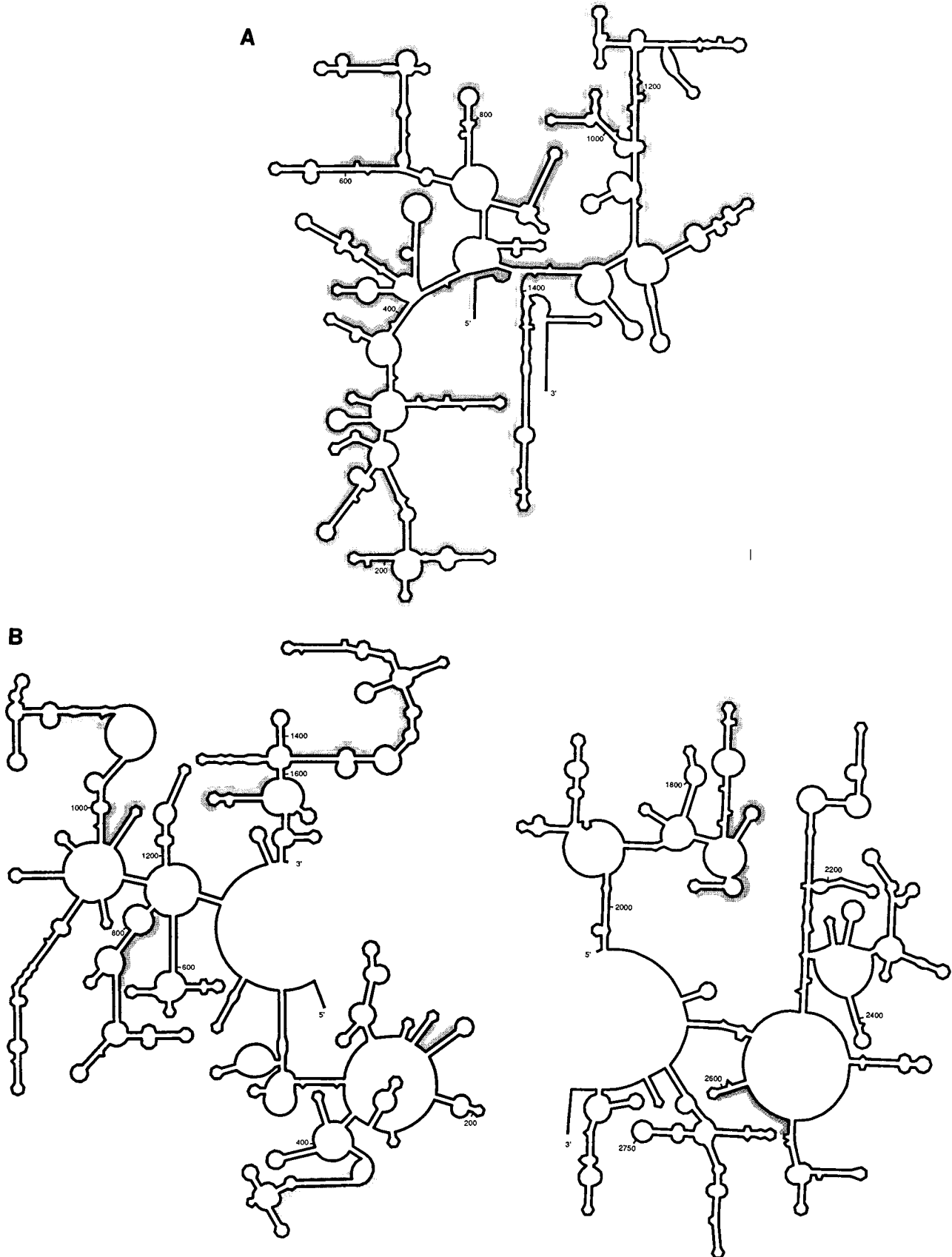


FIG. 9. Secondary-structure models of the bacterial 16S (A) and 23S (B) rRNA molecules. Stippled areas highlight target regions of successful whole-cell hybridizations. The numbers refer to *E. coli* numbering (22).

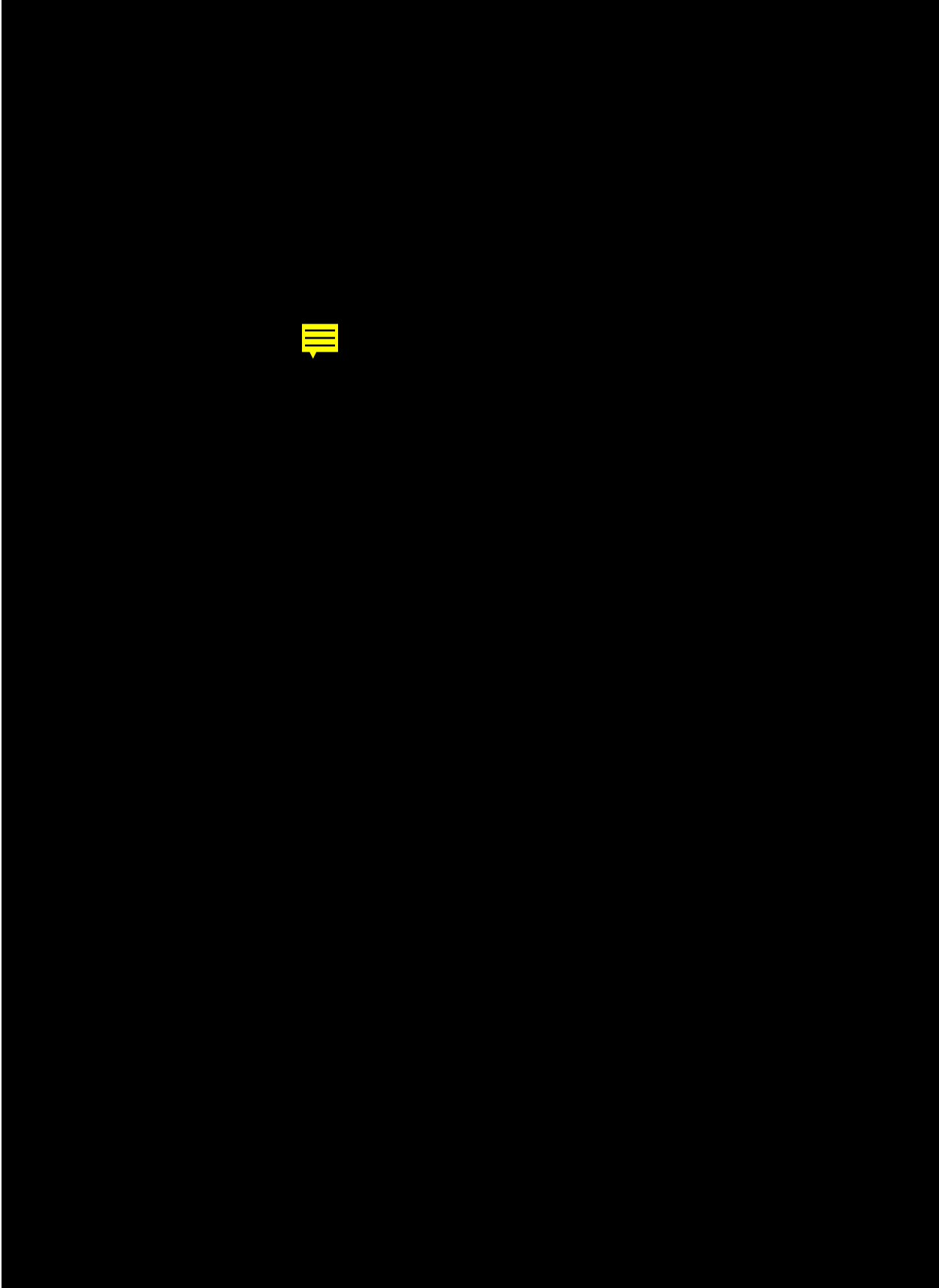


FIG. 10. (A) The left-hand panel shows intrinsic fluorescence reported with a rhodamine-specific filter set (also present with other filter sets) in an untreated root section of the tree fern *Ceratozamia mexicana* var. *longifolia*, which contains strongly autofluorescent cyanobacteria. The right-hand panel shows cyanobacteria detected in an ethanol-fixed section with a nonfluorescent assay involving a peroxidase-labeled oligonucleotide probe and the substrate diaminobenzidine, forming dark precipitates in the cells. Photomicrographs by B. Zarda. (B) Simultaneous three-color detection. A mixture of paraformaldehyde-fixed cells of *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, and *Acinetobacter* sp. was simultaneously hybridized with three polynucleotide transcript probes carrying multiple labels of aminomethylcoumarin, fluorescein, and digoxigenin (detected with rhodamine-labeled anti-digoxigenin antibody fragments). On a triple-exposure photomicrograph with Zeiss (Oberkochen, Germany) filter sets 01, 09, and 15, the three species can be distinguished by the colors blue, green, and red. Yellow spots are caused by an overlay of red and green cells. Photomicrographs by K. H. Trebesius. (C) Spatial distribution of cells hybridizing with probes specific for the beta (left-hand panel) and gamma (right-hand panel) subclasses of *Proteobacteria* in an activated-sludge sample. After hybridization, optical sectioning was performed at 1- μ m intervals with a Zeiss confocal laser scanning microscope. The results were transformed in red-green three-dimensional reconstructions. For a three-dimensional impression, red-green reconstructions must be viewed with appropriate red-green glasses. Data acquisition and processing on a Zeiss confocal laser scanning microscope were performed by P. Hutzler, B. Assmus, and M. Wagner.

2- μ m intervals, clearly showing a bacteria-covered fiber resting on a floc.

Finally, it has to be stressed that microscopy is just one analytical method to examine cells after whole-cell hybridization. If the sample consisted of suspended microorganisms, cell enumeration and quantification of the fluorescence would be much more rapidly achieved with a flow cytometer (1, 12, 15, 162). This technique could also be more easily automated.

FUTURE PERSPECTIVES

The rRNA approach, together with other molecular techniques (for a recent review, see reference 147), bears great

potential for an analysis of microbial diversity which is unbiased by the limits of pure-culture techniques. The results of one decade of research are still very fragmentary, but several important conclusions can already be drawn.

(i) Direct rDNA sequencing clearly indicates a huge undiscovered diversity within the domains *Bacteria* and *Archaea*. It will be a continuing task of microbiologists to identify, isolate, and characterize new organisms, in respect to both phylogeny and physiology. The rRNA approach will be an important tool in this effort. It will be helpful in monitoring the cultivation of hitherto uncultured bacteria. Its applications will soon extend from the more basic to the applied field. By making the screening for new microbial diversity more directed, the rRNA ap-

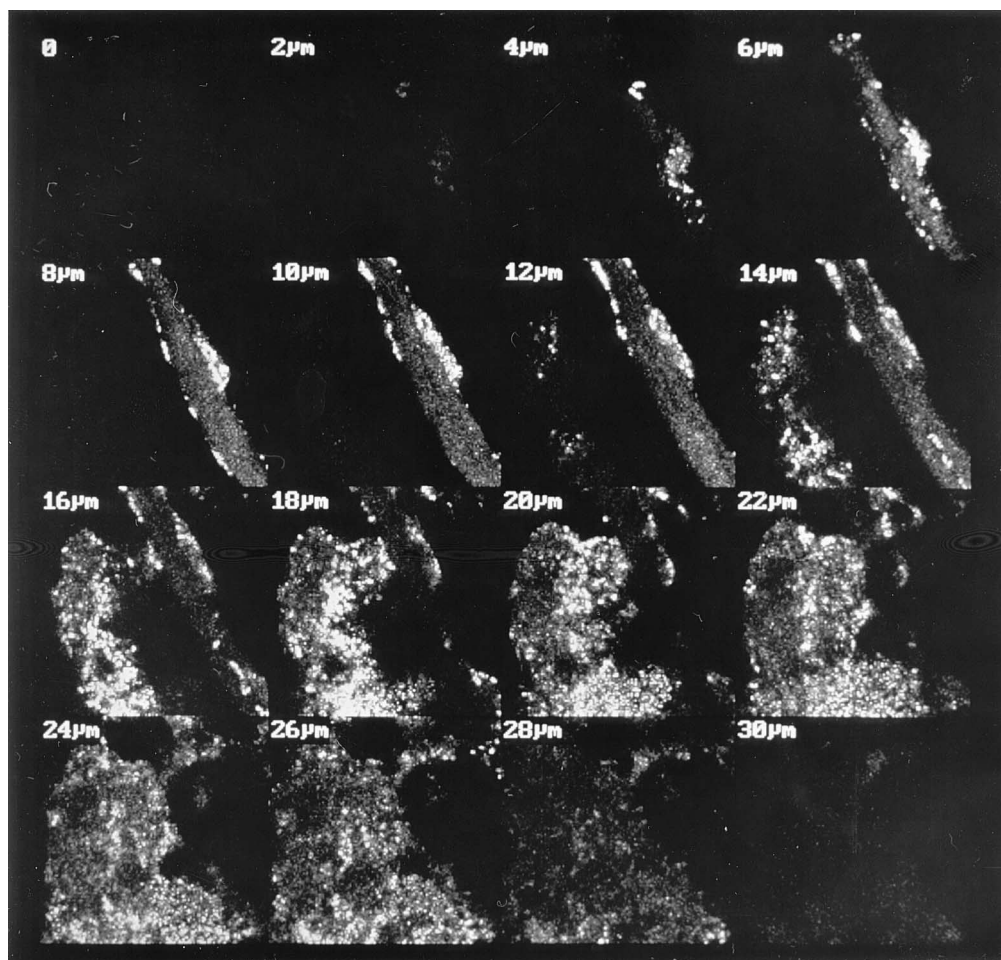


FIG. 11. The confocal laser scanning microscope data on the spatial distribution of beta subclass proteobacteria (corresponding to Fig. 10C, left) are presented as a series of 16 μ y sections at 2- μ m intervals.

proach will help to access additional genetic potential for biotechnology.

(ii) Revolutionary insights into ecology will be obtained by studying microorganisms in their niches. Correlations between the community composition, the spatial relationships of the different members, and the function of the populations can now be determined *in situ*. This is extremely important not only for an understanding of the very abundant symbioses but also for an understanding of most biogeochemical cycles, in which many transformations are catalyzed by consortia and not by single species of microorganisms.

(iii) With the advent of increasingly sensitive *in situ* hybridization techniques, detection of lower-copy-number nucleic acids will become possible. *In situ* monitoring of the lateral transfer of plasmids in natural environments and the detection of specific mRNA (68) are just two of many possible applications. The parallel probing for specific rRNA and mRNA molecules will help us to directly address specific *in situ* activities of defined populations.

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REFERENCES

- Amann, R. I. Unpublished observations.
- Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56**:1919–1925.
- Amann, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* **172**:762–770.
- Amann, R. I., C. Lin, R. Key, L. Montgomery, and D. A. Stahl. 1992. Diversity among *Fibrobacter* isolates: towards a phylogenetic and habitat-based classification. *Syst. Appl. Microbiol.* **15**:23–31.
- Amann, R., N. Springer, W. Ludwig, H.-D. Görtz, and K.-H. Schleifer. 1991. Identification and phylogeny of uncultured bacterial endosymbionts. *Nature (London)* **351**:161–164.
- Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl. 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl. Environ. Microbiol.* **58**:614–623.
- Amann, R. I., B. Zarda, D. A. Stahl, and K. H. Schleifer. 1992. Identification of individual prokaryotic cells with enzyme-labeled, rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **58**:3007–3011.
- Amikam, D., G. Glaser, and S. Razin. 1984. Mycoplasmas (*Mollicutes*) have a low number of rRNA genes. *J. Bacteriol.* **158**:376–378.
- Angert, E. R., K. D. Clements, and N. R. Pace. 1993. The largest bacterium. *Nature (London)* **362**:239–241.
- Arnoldi, J., C. Schlüter, M. Duchrow, L. Hübner, M. Ernst, A. Teske, H.-D. Flad, J. Gerdes, and E. C. Böttger. 1992. Species-specific assessment of *Mycobacterium leprae* in skin biopsies by *in situ* hybridization and polymerase chain reaction. *Lab. Invest.* **66**:618–623.
- Ashkin, A., J. M. Dziedzic, and T. Yamane. 1987. Optical trapping and manipulation of single cells using infrared laser beams. *Nature (London)* **330**:769–771.
- Aznar, R., W. Ludwig, R. Amann, and K. H. Schleifer. 1994. Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. *Int. J. Syst. Bacteriol.* **44**:330–337.
- Bauman, J. G. J., and P. Bentvelzen. 1988. Flow cytometric detection of ribosomal RNA in suspended cells by fluorescent *in situ* hybridization. *Cytometry* **9**:517–524.
- Baumann, P., M. A. Munson, C.-Y. Lai, M. A. Clark, L. Baumann, N. A. Moran, and B. C. Campbell. 1993. Origin and properties of bacterial endosymbionts of aphids, whiteflies, and mealybugs. *ASM News* **59**:21–24.
- Beimfohr, C., A. Krause, R. Amann, W. Ludwig, and K. H. Schleifer. 1993. *In situ* identification of lactococci, enterococci and streptococci. *Syst. Appl. Microbiol.* **16**:450–456.
- Bertin, B., O. Broux, and M. van Hoegarden. 1990. Flow cytometric detection of yeast by *in situ* hybridization with a fluorescent ribosomal RNA probe. *J. Microbiol. Methods* **12**:1–12.
- Betzl, D., W. Ludwig, and K. H. Schleifer. 1990. Identification of lactococci and enterococci by colony hybridization with 23S-rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **56**:2927–2929.
- Bohlool, B. B., and E. L. Schmidt. 1980. The immunofluorescence approach in microbial ecology. *Adv. Microb. Ecol.* **4**:203–241.
- Braun-Howland, E. B., S. A. Danielsen, and S. A. Nierzwicki-Bauer. 1992. Development of a rapid method for detecting bacterial cells *in situ* using 16S rRNA-targeted probes. *BioTechniques* **13**:928–933.
- Brenner, D. J., S. P. O'Connor, H. H. Winkler, and A. G. Steigerwalt. 1993. Proposal to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. *Int. J. Syst. Bacteriol.* **43**:777–786.
- Britschgi, T. B., and S. J. Giovannoni. 1991. Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. *Appl. Environ. Microbiol.* **57**:1707–1713.
- Brock, T. D. 1987. The study of microorganisms *in situ*: progress and problems. *Symp. Soc. Gen. Microbiol.* **41**:1–17.
- Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.* **148**:107–127.
- Bull, A. T., M. Goodfellow, and J. H. Slater. 1992. Biodiversity as a source of innovation in biotechnology. *Annu. Rev. Microbiol.* **46**:219–252.
- Bull, A. T., and D. J. Hardman. 1991. Microbial diversity. *Curr. Opin. Biotechnol.* **2**:421–428.
- Burggraf, S., T. Mayer, R. Amann, S. Schadhauer, C. R. Woese, and K. O. Stetter. 1994. Identifying *Archaea* with rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **60**:3112–3119.
- Button, D. K., F. Schut, P. Quang, R. Martin, and B. R. Robertson. 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. *Appl. Environ. Microbiol.* **59**:881–891.
- Cary, S. C., and S. J. Giovannoni. 1993. Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. *Proc. Natl. Acad. Sci. USA* **90**:5695–5699.
- Colwell, R. R., P. R. Brayton, D. J. Grimes, D. R. Roszak, S. A. Huq, and L. M. Palmer. 1985. Viable but nonculturable *Vibrio cholerae* and related pathogens in the environment: implications for the release of genetically engineered microorganisms. *Bio/Technology* **3**:817–820.
- Dasch, G. A., E. Weiss, and K.-P. Chang. 1984. Endosymbionts of insects, p. 811–833. *In* N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
- DeLong, E. F. 1992. *Archaea* in coastal marine environments. *Proc. Natl. Acad. Sci. USA* **89**:5685–5689.
- DeLong, E. F., R. B. Frankel, and D. A. Bazylinski. 1993. Multiple evolutionary origins of magnetotaxis in bacteria. *Science* **259**:803–806.
- DeLong, E. F., D. G. Franks, and A. L. Alldredge. 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol. Oceanogr.* **38**:924–934.
- DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single microbial cells. *Science* **243**:1360–1363.
- Deng, S., and C. Hiruki. 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *J. Microbiol. Methods* **14**:53–61.
- Devereux, R., M. D. Kane, J. Winfrey, and D. A. Stahl. 1992. Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. *Syst. Appl. Microbiol.* **15**:601–609.
- DiChristina, T. J., and E. F. DeLong. 1993. Design and application of rRNA-targeted oligonucleotide probes for the dissimilatory iron- and manganese-reducing bacterium *Shewanella putrefaciens*. *Appl. Environ. Microbiol.* **59**:4152–4160.
- Distel, D. L., E. F. DeLong, and J. B. Waterbury. 1991. Phylogenetic characterization and *in situ* localization of the bacterial symbiont of shipworms (Teredinidae: Bivalvia) by using 16S rRNA sequence analysis and oligodeoxynucleotide probe hybridization. *Appl. Environ. Microbiol.* **57**:2376–2382.
- Distel, D. L., D. J. Lane, G. J. Olsen, S. J. Giovannoni, B. Pace, N. R. Pace, D. A. Stahl, and H. Felbeck. 1988. Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences. *J. Bacteriol.* **170**:2506–2510.
- Distel, D. L., and A. P. Wood. 1992. Characterization of the gill symbiont of *Thyasira flexuosa* (Thyasiridae: Bivalvia) by use of polymerase chain reaction and 16S rRNA sequence analysis. *J. Bacteriol.* **174**:6317–6320.
- Drozanski, W. J. 1991. *Sarcobium lyticum* gen. nov., sp. nov., an obligate intracellular bacterial parasite of small free-living amoebae. *Int. J. Syst. Bacteriol.* **41**:82–87.
- Edman, J. C., J. A. Kovacs, H. Masur, D. V. Santi, H. J. Elwood, and M. L. Sogin. 1988. Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the fungi. *Nature (London)* **334**:519–522.

42. Ehrmann, M., W. Ludwig, and K. H. Schleifer. 1992. Species specific oligonucleotide probe for the identification of *Streptococcus thermophilus*. Syst. Appl. Microbiol. 15:453-455.
43. Embley, T. M., B. J. Finlay, and S. Brown. 1992. RNA sequence analysis shows that the symbionts in the ciliate *Metopus contortus* are polymorphs of a single methanogen species. FEMS Microbiol. Lett. 97:57-62.
44. Embley, T. M., B. J. Finlay, R. H. Thomas, and P. L. Dyal. 1992. The use of rRNA sequences and fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate *Metopus palaeformis* and its archaeobacterial endosymbiont. J. Gen. Microbiol. 138:1479-1487.
45. Fenchel, T., and C. Bernard. 1993. Endosymbiotic purple non-sulphur bacteria in an anaerobic ciliated protozoan. FEMS Microbiol. Lett. 110:21-25.
46. Fenchel, T., T. Perry, and A. Thane. 1977. Anaerobiosis and symbiosis with bacteria of free-living ciliates. J. Protozool. 24:154-163.
47. Fenchel, T., and N. B. Ramsing. 1992. Identification of sulfate reducing ectosymbiotic bacteria from anaerobic ciliates using 16S rRNA binding oligonucleotide probes. Arch. Microbiol. 158:394-397.
48. Ferguson, R. L., E. N. Buckley, and A. V. Palumbo. 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. 47:49-55.
49. Finlay, B. J., T. M. Embley, and T. Fenchel. 1993. A new polymorphic methanogen, closely related to *Methanococcus parvum*, living in stable symbiosis within the anaerobic ciliate *Trimyema* sp. J. Gen. Microbiol. 139:371-378.
50. Fishelson, L., W. L. Montgomery, and A. A. Myrberg, Jr. 1985. A unique symbiosis in the gut of tropical herbivorous surgeonfish (Acanthuridae: Teleostei) from the Red Sea. Science 229:49-51.
51. Flårdh, K., P. S. Cohen, and S. Kjelleberg. 1992. Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. J. Bacteriol. 174:6780-6788.
52. Fletcher, M., and K. C. Marshall. 1982. Are solid surfaces of ecological significance to aquatic bacteria? Adv. Microb. Ecol. 6:199-236.
53. Fox, G. E., J. D. Wisotzky, and P. Jurtschuk, Jr. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42:166-170.
54. Fry, N. K., T. J. Rowbotham, N. A. Saunders, and T. M. Embley. 1991. Direct amplification and sequencing of 16S ribosomal DNA of an intracellular *Legionella* species recovered by amoebal enrichment from the sputum of a patient with pneumonia. FEMS Microbiol. Lett. 83:165-168.
55. Fuhrman, J. A., K. McCallum, and A. A. Davis. 1992. Novel major archaeobacterial group from marine plankton. Nature (London) 356:148-149.
56. Fuhrman, J. A., K. McCallum, and A. A. Davis. 1993. Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific oceans. Appl. Environ. Microbiol. 59:1294-1302.
57. Gebhart, C. J., S. M. Barns, S. McOrist, G.-F. Lin, and G. H. K. Lawson. 1993. Ileal symbiont intracellularis, an obligate intracellular bacterium of porcine intestines showing a relationship to *Desulfovibrio* species. Int. J. Syst. Bacteriol. 43:533-538.
58. Gersdorf, H., A. Meissner, K. Pelz, G. Krekeler, and U. B. Göbel. 1993. Identification of *Bacteroides forsythus* in subgingival plaque from patients with advanced periodontitis. J. Clin. Microbiol. 31:941-946.
59. Gersdorf, H., K. Pelz, and U. B. Göbel. 1993. Fluorescence in situ hybridization for direct visualization of Gram-negative anaerobes in subgingival plaque samples. FEMS Immunol. Med. Microbiol. 6:109-114.
60. Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field. 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) 345:60-63.
61. Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace. 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. 170:720-726.
62. Giovannoni, S. J., E. F. DeLong, T. M. Schmidt, and N. R. Pace. 1990. Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl. Environ. Microbiol. 56:2572-2575.
63. Goebel, U. B., A. Geiser, and E. J. Stanbridge. 1987. Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. J. Gen. Microbiol. 133:1969-1974.
64. Görtz, H.-D. 1983. Endonuclear symbionts in ciliates. Int. Rev. Cytol. 14(Suppl.):145-176.
65. Görtz, H.-D. 1986. Endonucleobiosis in ciliates. Int. Rev. Cytol. 102:169-213.
66. Haase, A., M. Brahic, L. Stowring, and H. Blum. 1984. Detection of viral nucleic acids by *in situ* hybridization. Methods Virol. 7:189-226.
67. Hahn, D., R. I. Amann, W. Ludwig, A. D. L. Akkermans, and K.-H. Schleifer. 1992. Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. J. Gen. Microbiol. 138:879-887.
68. Hahn, D., R. I. Amann, and J. Zeyer. 1993. Detection of mRNA in *Streptomyces* cells by *in situ* hybridization with digoxigenin-labeled probes. Appl. Environ. Microbiol. 59:2753-2757.
69. Hahn, D., R. I. Amann, and J. Zeyer. 1993. Whole cell hybridization of *Frankia* strains with fluorescence- or digoxigenin-labeled, 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. 59:1709-1716.
70. Haygood, M. G., and D. L. Distel. 1993. Bioluminescent symbionts of flashlight fishes and deepsea anglerfishes form unique lineages related to the genus *Vibrio*. Nature (London) 363:154-156.
71. Heckmann, K., and H. J. Schmidt. 1987. *Polynucleobacter necessarius* gen. nov., sp. nov., an obligately endosymbiotic bacterium living in the cytoplasm of *Euplotes aediculatus*. Int. J. Syst. Bacteriol. 37:456-457.
72. Hicks, R., R. I. Amann, and D. A. Stahl. 1992. Dual staining of natural bacterioplankton with 4',6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. 58:2158-2163.
73. Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. König, R. Rachel, I. Rockinger, H. Fricke, and K. O. Stetter. 1992. *Aquifex pyrophilus* gen. nov. sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst. Appl. Microbiol. 15:340-351.
74. Jarvis, E. D., R. L. Widom, G. LaFauci, Y. Setoguchi, I. R. Richter, and R. Rudner. 1988. Chromosomal organization of rRNA operons in *Bacillus subtilis*. Genetics 120:625-635.
75. Jones, J. G. 1977. The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures. Freshwater Biol. 7:67-91.
76. Jurtschuk, R. J., M. Blick, J. Bresser, G. E. Fox, and P. Jurtschuk. 1992. Rapid *in situ* hybridization technique using 16S rRNA segments for detecting and differentiating the closely related gram-positive organisms *Bacillus polymyxa* and *Bacillus macerans*. Appl. Environ. Microbiol. 58:2571-2578.
77. Kane, M. D., L. K. Poulsen, and D. A. Stahl. 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl. Environ. Microbiol. 59:682-686.
78. Kemp, P. F., S. Lee, and J. LaRoche. 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl. Environ. Microbiol. 59:2594-2601.
79. Kerkhof, L., and B. B. Ward. 1993. Comparison of nucleic acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth rate in a marine bacterium. Appl. Environ. Microbiol. 59:1303-1309.
80. Khalfan, H., R. Abuknesha, M. Rand-Weaver, R. G. Price, and D. Robinson. 1986. Aminomethyl coumarin acetic acid: a new fluorescent labelling agent for proteins. Histochem. J. 18:497-499.
81. Kogure, K., U. Simidu, and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25:415-420.
82. Kogure, K., U. Simidu, and N. Taga. 1980. Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol. 26:318-323.
83. Kramer, J. G., and F. L. Singleton. 1993. Measurement of rRNA variations in natural communities of microorganisms on the southeastern U.S. continental shelf. Appl. Environ. Microbiol. 59:2430-2436.
84. Lane, D. J., D. A. Stahl, G. J. Olsen, D. J. Heller, and N. R. Pace. 1985. Phylogenetic analysis of the genera *Thiobacillus* and *Thiomicrospira* by 5S rRNA sequences. J. Bacteriol. 163:75-81.
85. LaRiviere, J. W. M., and K. Schmidt. 1991. Morphologically conspicuous sulfur-oxidizing eubacteria, p. 3934-3947. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.
86. Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese. 1993. The ribosomal database project. Nucleic Acids Res. 21:3021-3023.
87. Lee, S., C. Malone, and P. F. Kemp. 1993. Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Mar. Ecol. Prog. Ser. 101:193-201.
88. Liesack, W., and E. Stackebrandt. 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. 174:5072-5078.
89. Liesack, W., H. Weyland, and E. Stackebrandt. 1991. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb. Ecol. 21:191-198.
90. Lim, E. L., L. A. Amaral, D. A. Caron, and E. F. DeLong. 1993. Application of rRNA-based probes for observing marine nanoplanctonic protists. Appl. Environ. Microbiol. 59:1647-1655.
- 90a. Ludwig, W. Unpublished observations.
91. Mann, S., N. H. C. Sparks, and R. G. Board. 1990. Magnetotactic bacteria: microbiology, biomineralization, palaeomagnetism, and biotechnology. Adv. Microb. Physiol. 31:125-181.
92. Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15:593-600.
93. Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K. H. Schleifer. Whole cell hybridization probes for members of the cytophaga-flexibacterium-bacteroides (CFB) phylum. Submitted for publication.
94. Manz, W., R. Amann, R. Szewzyk, U. Szewzyk, T.-A. Stenström, P. Hutzler, and K. H. Schleifer. *In situ* identification of *Legionellaceae* using 16S rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. Microbiology, in press.
95. Manz, W., U. Szewzyk, P. Eriksson, R. Amann, K. H. Schleifer, and T.-A.

- Stenström. 1993. *In situ* identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. *Appl. Environ. Microbiol.* **59**:2293–2298.
96. McSweeney, C. S., R. I. Mackie, A. A. Odenyo, and D. A. Stahl. 1993. Development of an oligonucleotide probe targeting 16S rRNA and its application for detection and quantification of the ruminal bacterium *Synergistes jonesii* in a mixed-population chemostat. *Appl. Environ. Microbiol.* **59**:1607–1612.
97. Moench, T. T., and W. A. Konetzka. 1978. A novel method for the isolation and study of a magnetotactic bacterium. *Arch. Microbiol.* **119**:203–212.
98. Moran, N. A., M. A. Munson, P. Baumann, and H. Ishikawa. 1993. A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proc. R. Soc. London Ser. B* **253**:167–171.
99. Munson, M. A., P. Baumann, M. A. Clark, L. Baumann, N. A. Moran, D. J. Voegtlin, and B. C. Campbell. 1991. Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *J. Bacteriol.* **173**:6321–6324.
100. Munson, M. A., P. Baumann, and M. G. Kinsey. 1991. *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. *Int. J. Syst. Bacteriol.* **41**:566–568.
101. Muzyer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695–700.
102. Namba, S., H. Oyaizu, S. Kato, S. Iwanami, and T. Tsuchizaki. 1993. Phylogenetic diversity of phytopathogenic mycoplasma-like organisms. *Int. J. Syst. Bacteriol.* **43**:461–467.
103. Neef, J. M., Y. Vandepoer, P. DeRijk, S. Chapelle, and R. DeWachter. 1993. Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Res.* **21**:3025–3049.
104. Neimark, H., and B. C. Kirkpatrick. 1993. Isolation and characterization of full-length chromosomes from non-culturable plant-pathogenic Mycoplasma-like organisms. *Mol. Microbiol.* **7**:21–28.
105. Oliver, J. D., L. Nilsson, and S. Kjelleberg. 1991. Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. *Appl. Environ. Microbiol.* **57**:2640–2644.
106. Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl. 1986. Microbial ecology and evolution: a ribosomal RNA approach. *Annu. Rev. Microbiol.* **40**:337–365.
107. Olsen, G. J., and C. R. Woese. 1993. Ribosomal RNA: a key to phylogeny. *FASEB J.* **7**:113–123.
108. O'Neill, S. L., R. Giordano, A. M. E. Colbert, T. L. Karr, and H. M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. USA* **89**:2699–2702.
- 108a. Petersen, N. L. Personal communication.
109. Polz, M., D. L. Distel, B. Zarda, R. Amann, H. Felbeck, J. A. Ott, and C. M. Cavanaugh. 1993. Phylogenetic analysis of a highly specific association between ectosymbiotic sulfur-oxidizing bacteria and marine nematodes. *Appl. Environ. Microbiol.* **60**:4461–4467.
110. Porter, K. G., and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25**:943–948.
111. Poulsen, L. K., G. Ballard, and D. A. Stahl. 1993. Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. *Appl. Environ. Microbiol.* **59**:1354–1360.
112. Ramsing, N. B., M. Kühl, and B. B. Jörgensen. 1993. Distribution of sulfate-reducing bacteria, O₂ and H₂S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. *Appl. Environ. Microbiol.* **59**:3820–3849.
113. Raskin, L., L. K. Poulsen, D. R. Noguera, B. E. Rittmann, and D. A. Stahl. 1994. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* **60**:1241–1248.
114. Raskin, L., J. M. Stromley, B. E. Rittmann, and D. A. Stahl. 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.* **60**:1232–1240.
115. Razin, S. 1991. The genera *Mycoplasma*, *Ureaplasma*, *Acholeplasma*, *Anaeroplasmata*, and *Asteroplasmata*, p. 1937–1959. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, New York.
116. Regnery, R. L., B. E. Anderson, J. E. Clarridge III, M. C. Rodriguez-Barradas, D. C. Jones, and J. H. Carr. 1992. Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. *J. Clin. Microbiol.* **30**:265–274.
117. Rehnstam, A.-S., S. Bäckman, D. C. Smith, F. Azam, and A. Hagström. 1993. Blooms of sequence-specific culturable bacteria in the sea. *FEMS Microbiol. Ecol.* **102**:161–166.
118. Relman, D. A., P. W. Lepp, K. N. Sadler, and T. M. Schmidt. 1992. Phylogenetic relationships among the agent of bacillary angiomatosis, *Bartonella bacilliformis* and other alpha-proteobacteria. *Mol. Microbiol.* **6**:1801–1807.
119. Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins. 1990. The agent of bacillary angiomatosis: an approach to the identification of uncultured pathogens. *N. Engl. J. Med.* **323**:1573–1580.
120. Relman, D. A., T. M. Schmidt, R. P. MacDermott, and S. Falkow. 1992. Identification of the uncultured bacillus of Whipple's disease. *N. Engl. J. Med.* **327**:293–301.
121. Reysenbach, A.-L., L. J. Giver, G. S. Wickham, and N. R. Pace. 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* **58**:3417–3418.
122. Roller, C., M. Wagner, R. Amann, W. Ludwig, and K. H. Schleifer. 1994. *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiology* **140**:2849–2858.
123. Roszak, D. B., and R. R. Colwell. 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* **51**:365–379.
124. Roszak, D. B., D. J. Grimes, and R. R. Colwell. 1984. Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. *Can. J. Microbiol.* **30**:334–338.
125. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Ehrlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487–491.
126. Salama, M., W. Sandine, and S. Giovannoni. 1991. Development and application of oligonucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris*. *Appl. Environ. Microbiol.* **57**:1313–1318.
127. Schaechter, M. O., O. Maaloe, and N. O. Kjeldgaard. 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. *J. Gen. Microbiol.* **19**:592–606.
128. Schleifer, K. H., R. Amann, W. Ludwig, C. Rothemund, N. Springer, and S. Dorn. 1992. Nucleic acid probes for the identification and *in situ* detection of pseudomonads, p. 127–134. In E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas: molecular biology and biotechnology*. American Society for Microbiology, Washington, D.C.
129. Schleifer, K. H., W. Ludwig, R. Amann, C. Hertel, M. Ehrmann, W. Köhler, and A. Krause. 1991. Phylogenetic relationships of lactic acid bacteria and their identification with nucleic acid probes, p. 23–32. In G. Novel and J.-F. Le Querler (ed.), *Actes du colloque LACTIC 91*. Caen University Press, Caen, France.
130. Schleifer, K. H., D. Schüler, S. Spring, M. Weizenegger, R. Amann, W. Ludwig, and M. Köhler. 1991. The genus *Magnetospirillum* gen. nov. Description of *Magnetospirillum gryphiswaldense* sp. nov. and transfer of *Aquaspirillum magnetotacticum* to *Magnetospirillum magnetotacticum* comb. nov. *Syst. Appl. Microbiol.* **14**:379–385.
131. Schleifer, K. H., and E. Stackebrandt. 1983. Molecular systematics of prokaryotes. *Annu. Rev. Microbiol.* **37**:143–187.
132. Schloter, M. R. Borlinghaus, W. Bode, and A. Hartmann. 1993. Direct identification, and localization of Azospirillum in the rhizosphere of wheat using fluorescence-labelled monoclonal antibodies and confocal scanning laser microscopy. *J. Microsc.* **171**:173–177.
133. Schmidt, T. M., E. F. DeLong, and N. R. Pace. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. *J. Bacteriol.* **173**:4371–4378.
134. Schut, F., E. J. DeVries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button. 1993. Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. *Appl. Environ. Microbiol.* **59**:2150–2160.
135. Southwick, P. L., L. A. Ernst, E. W. Tauriello, S. R. Parker, R. B. Mujumdar, S. R. Mujumdar, H. A. Clever, and A. S. Waggoner. 1990. Cyanine dye labeling reagents—carboxymethylindocyanine succinimidyl esters. *Cytometry* **11**:418–430.
136. Spring, S., R. Amann, W. Ludwig, K.-H. Schleifer, and N. Petersen. 1992. Phylogenetic diversity and identification of nonculturable magnetotactic bacteria. *Syst. Appl. Microbiol.* **15**:116–122.
137. Spring, S., R. Amann, W. Ludwig, K. H. Schleifer, H. van Gernerden, and N. Petersen. 1993. Dominating role of an unusual magnetotactic bacterium in the microaerophilic zone of a freshwater sediment. *Appl. Environ. Microbiol.* **59**:2397–2403.
138. Springer, N. 1992. Phylogenie und *in-situ* Nachweis nicht-kultivierbarer bakterieller Endosymbionten, Ph.D. thesis. Technische Universität München, Munich.
139. Springer, N., W. Ludwig, R. Amann, H. J. Schmidt, H.-D. Görtz, and K.-H. Schleifer. 1993. Occurrence of fragmented 16S rRNA in an obligate bacterial endosymbiont of *Paramecium caudatum*. *Proc. Natl. Acad. Sci. USA* **90**:9892–9895.
140. Springer, N., W. Ludwig, V. Drozanski, R. Amann, and K.-H. Schleifer. 1992. The phylogenetic status of *Sarcobium lyticum*, an obligate intracellular bacterial parasite of small amoebae. *FEMS Microbiol. Lett.* **96**:199–202.
141. Stackebrandt, E., W. Liesack, and B. M. Goebel. 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB J.* **7**:232–236.
142. Stahl, D. A. 1988. Phylogenetically based studies of microbial ecosystem perturbations, p. 373–390. In P. Hedin, J. J. Menn, and R. M. Hollingsworth (ed.), *American Chemical Society symposium volume*. Biotechnology for crop protection. American Chemical Society, Washington, D.C.

143. **Stahl, D. A.** 1986. Evolution, ecology, and diagnosis: unity in variety. *Bio/Technology* **4**:623–628.
144. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes in bacterial systematics, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons Ltd. Chichester, England.
145. **Stahl, D. A., R. Devereux, R. I. Amann, B. Flesher, C. Lin, and J. Stromley.** 1989. Ribosomal RNA based studies of natural microbial diversity and ecology, p. 669–673. *In* T. Hattori, Y. Ishida, Y. Maruyama, R. Morita, and A. Uchida (ed.), *Recent advances in microbial ecology*. Japan Scientific Societies Press, Tokyo, Japan.
146. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* **54**:1079–1084.
147. **Stahl, D. A., and M. D. Kane.** 1992. Methods of microbial identification, tracking and monitoring of function. *Curr. Opin. Biotechnol.* **3**:244–252.
148. **Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace.** 1984. Analysis of hydrothermal vent-associated symbionts by ribosomal RNA sequences. *Science* **224**:409–411.
149. **Stahl, D. A., D. J. Lane, G. J. Olsen, and N. R. Pace.** 1985. Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. *Appl. Environ. Microbiol.* **49**:1379–1384.
150. **Staley, J. T., and A. Konopka.** 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu. Rev. Microbiol.* **39**:321–346.
151. **Stouthamer, R., J. A. J. Breeuwer, R. F. Luck, and J. H. Werren.** 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature (London)* **361**:66–68.
152. **Szewzyk, U., W. Manz, R. Amann, K.-H. Schleifer, and T.-A. Stenström.** 1994. Growth and in situ detection of a pathogenic *Escherichia coli* in biofilms of a heterotrophic water bacterium by use of 16S- and 23S-rRNA-directed fluorescent oligonucleotide probes. *FEMS Microbiol. Ecol.* **13**:169–175.
153. **Torsvik, V., J. Goksoyr, and F. L. Daae.** 1990. High diversity of DNA of soil bacteria. *Appl. Environ. Microbiol.* **56**:782–787.
154. **Trebesius, K., R. Amann, W. Ludwig, K. Mühlegger, and K. H. Schleifer.** Identification of whole fixed bacterial cells with nonradioactive rRNA-targeted transcript probes. *Appl. Environ. Microbiol.* **60**:3228–3235.
155. **Tsien, H. C., B. J. Bratina, K. Tsuji, and R. S. Hanson.** 1990. Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylotrophic bacteria. *Appl. Environ. Microbiol.* **56**:2858–2865.
156. **Tully, J. G., and R. F. Whitcomb.** 1991. The genus *Spiroplasma*, p. 1960–1980. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, New York.
157. **Unterman, B. M., P. Baumann, and D. L. McLean.** 1989. Pea aphid symbiont relationships established by analysis of the 16S rRNAs. *J. Bacteriol.* **171**:2970–2974.
158. **Van Bruggen, J. J. A., C. K. Stumm, and G. D. Vogels.** 1983. Symbiosis of methanogenic bacteria and sapropepic protozoa. *Arch. Mikrobiol.* **136**:89–95.
159. **Wagner, M., R. Amann, P. Kämpfer, B. Assmus, A. Hartmann, P. Hutzler, N. Springer, and K.-H. Schleifer.** 1994. Identification and in situ detection of gram-negative filamentous bacteria in activated sludge. *Syst. Appl. Microbiol.* **17**:405–417.
160. **Wagner, M., R. Amann, H. Lemmer, and K. H. Schleifer.** 1993. Probing activated sludge with proteobacteria-specific oligonucleotides: inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* **59**:1520–1525.
161. **Wagner, M., R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi, and K. H. Schleifer.** 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. *Appl. Environ. Microbiol.* **60**:792–800.
162. **Wallner, G., R. Amann, and W. Beisker.** 1993. Optimizing fluorescent in situ hybridization of suspended cells with rRNA-targeted oligonucleotide probes for the flow cytometric identification of microorganisms. *Cytometry* **14**:136–143.
163. **Ward, D. M., M. M. Bateson, R. Weller, and A. L. Ruff-Roberts.** 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. *Adv. Microb. Ecol.* **12**:219–286.
164. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature (London)* **345**:63–65.
165. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **37**:463–464.
166. **Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J. Bacteriol.* **171**:6455–6467.
167. **Weller, R., and D. M. Ward.** 1989. Selective recovery of 16S rRNA sequences from natural microbial communities in the form of cDNA. *Appl. Environ. Microbiol.* **55**:1818–1822.
168. **Werren, J. H., G. D. D. Hurst, W. Zhang, J. A. J. Breeuwer, R. Stouthamer, and M. E. N. Majerus.** 1994. Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). *J. Bacteriol.* **176**:388–394.
169. **Whatley, J. M., and C. Chapman-Andresen.** 1989. *Phylum Karyoblastea*, p. 167–185. *In* L. Margulis, J. Corliss, M. Melkonian, and D. Chapman (ed.), *Handbook of Protozoa*. Jones and Bartlett Publishers, Boston.
170. **Wilson, K. H., R. Blitchington, R. Frothingham, and J. A. Wilson.** 1991. Phylogeny of the Whipple's-disease-associated bacterium. *Lancet* **338**:474–475.
171. **Woese, C. R.** 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–271.
172. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. *Proc. Natl. Acad. Sci. USA* **87**:4576–4579.
173. **Woese, C. R., J. Maniloff, and L. B. Zablen.** 1980. Phylogenetic analysis of the mycoplasmas. *Proc. Natl. Acad. Sci. USA* **77**:494–498.
174. **Woese, C. R., E. Stackebrandt, and W. Ludwig.** 1985. What are mycoplasmas: the relationship of tempo and mode in bacterial evolution. *J. Mol. Evol.* **21**:305–316.
175. **Woese, C. R., E. Stackebrandt, T. J. Macke, and G. E. Fox.** 1985. A phylogenetic definition of the major eubacterial taxa. *Syst. Appl. Microbiol.* **6**:143–151.
176. **Wolin, M. J.** 1979. The rumen fermentation: a model for microbial interaction in anaerobic ecosystems. *Adv. Microb. Ecol.* **3**:49–77.
177. **Yang, D., Y. Oyaizu, H. Oyaizu, G. J. Olsen, and C. R. Woese.** 1985. Mitochondrial origins. *Proc. Natl. Acad. Sci. USA* **82**:4443–4447.
- 177a. **Zarda, B.** Unpublished data.
178. **Zarda, B., R. Amann, G. Wallner, and K. H. Schleifer.** 1991. Identification of single bacterial cells using digoxigenin-labelled, rRNA-targeted oligonucleotides. *J. Gen. Microbiol.* **137**:2823–2830.

