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Review article.
Identifying Culturable and Uncultured Prokaryotes

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Abstract

Recent advances in molecular microbial ecology have provided microbiologists with a wealth of information on the extent of phylogenetic relatedness among cultured organisms and the composition of microbial communities. However, in order to understand microbial ecology, more is needed than just the determination of novel types of organisms. Another important factor in the reconstruction of the foodweb as a driving force in ecosystem function is the knowledge of biochemical properties and/or the presence of genes coding for enzymes involved in biochemical pathways of isolated and non-cultured strains. While the structure of an environmental sample can in principle now be elucidated by determination of the intraspecific relationships between microorganisms, methods that would explore species richness and species abundance, as well as functional dependence between organisms and between the organisms and the organic and inorganic components of the sample are less well explored.

Keywords: Microbial diversity, 165 rDNA, gene sequences, oligonucleotide probes

1. Introduction

This brief communication will summarize the findings of studies analyzing environmental samples in order to determine the identity of prokaryotic organisms. The interested reader is referred to the original literature and to

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excellent reviews that have been published on the most widely analysed molecule, the 16S ribosomal (r) RNA/DNA from the viewpoint of elucidation of phylogenetic relationships of pure cultures (Woese, 1987), and the detection of species and higher taxa within environmental samples by oligonucleotide probing (Amann et al., 1995). Less information is scattered in the literature about the use of functional genes to obtain information about the biochemical activity within an environment and about the potential of this sample to react towards stress factors. These topics will not be covered here.

2. Determination of the Uniqueness of a New Isolate

Complete or almost complete 16S rDNA sequences are now available for more than 80% of all validly described prokaryotic species, and it can be assumed that more than 95% of these will have been analyzed within the next two years. The sequences are available from data banks such as EMBL or GenBank or can be obtained from the ribosomal data base Project (RDP, Maidak et al., 1993). The neophyte, who is not familiar with sequence alignment, selection of phylogenetically meaningful stretches and treeing algorithms, may take advantage of the service of RDP and NCBI, Bethesda, Maryland, USA (via recipon@ncbi.nlm.nih.gov) to determine the nearest phylogenetic neighbor free of charge via electronic media.

Many genera are analysed thoroughly by now in that 16S rDNA sequences of almost all species have been investigated. These studies have been useful for determining the taxonomic limitations of the 16S rDNA to unravel high relationships at the species level (Stackebrandt and Goebel, 1994). Percent similarity cannot be used to clearly delimitate species because many species share identical or virtually identical sequences, while DNA-DNA similarity values for these species are below 70%. Comparison of DNA-DNA similarity values, as obtained from hybridization of genomes, and 16S rDNA similarity values, as obtained from sequence analysis, revealed that the correlation curve of the two values is curvilinear. Above about 97 to 98% 16S rDNA sequence identity the corresponding total DNA similarities may cover the full range between 10 to 20% similarity to 90 to 100% similarity. However, below the rDNA identity value of 97% it is unlikely that the relatedness between genomes, as measured by hybridization, is as high as 70%. Below 96% rDNA identity the corresponding DNA similarity is about background level. It is therefore strongly recommended to perform DNA-DNA hybridization for those pairs of organisms that share more than 97% 16S rDNA identity while experience shows that below this level strains belong to different species.

It is apparent that sequence analysis is more objective than the several DNA-DNA reassociation methods, that are somewhat exposed to methodological bias. Nevertheless, the current definition of a species relies heavily of the results of DNA pairing, for historical reasons. The main advantage of having a complete 16S rDNA sequence of the type strain (and optimally from at least two more strains) of the species is the rapid allocation of this taxon within the radiation of prokaryotic species. The use of a conservative molecule of the size of 16S or 23S rDNA, that is extremely ancient in evolutionary terms, stable in function and unlikely to be involved in genetic exchange, is a solid basis for the objective phylogenetic placement – irrespective of morphology, colony color, motility, chemotaxonomy and other properties used in the past for describing taxa. Once the closest relatives are known, one can address questions of taxon allocation, such as: is it necessary to perform DNA reassociation (above 97% rDNA similarity), and do the neighbors differ in taxonomically sound properties to support species differentiation, and what are the genus- and family specific characters? However, the order at which genera and species diverged from each other can not always be decided with confidence (the relationship between *Photorhabdus* and *Xenorhabdus* is such an example [Rainey et al., 1995]). In the end, it is predominantly the presence or absence of distinguishing phenotypic characters that will dictate the naming of new taxa. In the absence of these properties it is recommended not to propose a formal description but to await the results of additional investigations of geno-and phenotypic characters (Wayne et al., 1987). On the other hand, we may find examples in which high relationship at the overall DNA level and the rDNA level is not an indication for close phenetic similarities. For example, two species of *Micrococcus* have high genetic relatedness, yet differ to such an extent in chemotaxonomic properties that they could be considered members of different genera. Except for *M. luteus* and *M. lylae* the genus is phylogenetically very heterogeneous. These two species, however, show 40% DNA-DNA similarity, the 16S rDNA identity is 96% and the transformation rate of auxotrophic markers is as high as 10%. However, the species differ in their peptidoglycan type, and in the composition of their menaquinones and polar lipids – characters used in the delineation of neighboring taxa such as *Arthrobacter*, *Cellulomonas* and related taxa (Stackebrandt et al., 1995). One could argue that despite their high genetic relatedness, these two species represent two different genera.

This example demonstrates that decisions about taxon allocation should not be based on a single character, even if we are confident that this character is superior to others that have failed in the past. The buzz-word in systematics is "polyphasic taxonomy", a term that has been proposed to indicate that a balanced selection of geno- and phenotypic characters should form the basis for

the description, rather than the emphasis on a few apparently meaningful ones, or the uncritical use of many characters, including superficial and genetically unstable properties.

3. Analysis of the Extent of Prokaryotic Diversity in Environmental Samples

Several strategies for analyzing environmental rDNA and rRNA sequences have been published, including shotgun cloning of total population DNA (Pace et al., 1985; Schmidt et al., 1991), reverse transcriptase of total 16S rRNA to generate cDNA (Weller and Ward, 1989; Ward et al., 1990) and the use of the PCR technology and specific primers to selectively amplify rRNA genes (DeLong, 1992; Embley et al., 1992a; Weisburg et al., 1991). Each strategy has strengths and weaknesses, and it should be remembered that each step in the procedure may influence the quantitative and qualitative recovery of sequences. Biases may occur from poor sampling methodology, or from insufficient lysis which may leave cells intact, such as many Gram-positive bacteria, yeasts, Archaea or microbial spores. All of the methods require that a gene library be produced in a cloning host, and it must also be remembered that the composition of the library can be greatly influenced by the cloning system itself. Variations in the restriction or amplification efficiencies of different environmental DNA's are likely to influence library composition. It has also been reported that archaeal rDNA sequences were rapidly lost from a gene library in *E. coli* (Fuhrman et al., 1993), and this may also be found for other phylogenetically distant sequences. It should also be kept in mind that the sequencing of small numbers of randomly chosen clones is unlikely to accurately reflect the statistical distribution of sequences within the library. It is likely that minor components are missed, either because only a small fraction of clones are analyzed, or because major components are overrepresented in the clone library due to bias in the initial enzymatic recovery of rRNA sequences and in the PCR reaction. The importance of minor components of a microbial community should not be underestimated because they may be ecologically important, as they provide a reservoir of capabilities which allows the system to respond to changing environmental conditions. Application of the MPN methods for DNA and PCR fragments may solve part of the problems.

Irrespective of the approach used to generate a library, it must then be screened to identify clones containing different rRNA genes. This can be done by regular sequence analysis, by single lane sequencing of individual clones to produce a bar-code representing the positions of a single dideoxy-nucleotide (Schmidt et al., 1991), by restriction mapping of DNA extracted from

individual clones (Giovannoni, 1991), or by probing using taxon-specific oligonucleotide probes (Britschgi and Giovannoni, 1991). In order to identify the phylogenetic affiliations, unique clones can then be fully sequenced and compared to sequences from reference taxa. The accuracy by which this can be done depends on the amount of sequence data available from the clones and on the choice and number of reference sequences. Time and financial constraints may explain that only fragments of the almost complete rRNA genes are sequenced from the clones and that analysis of short stretches is not always as reliable as that based on full sequences. Another problem for future comparison of results from different environmental studies is the incompleteness of sequences of the databases of environmental studies. Different groups have analysed different stretches of rDNA which allows their comparison to the database of cultured organisms for which full sequences are available, but which excludes analysis of the full range of environmental sequences.

4. Results of Community Analysis using 16S rRNA Sequences

The following lists some of the environments which have been studied with respect to the composition of natural microbial communities using rDNA/RNA sequences and summarizes the most important findings of the published data. Oceans: Schmidt et al. (1991); Giovannoni et al. (1990); Britschgi and Giovannoni (1991); De Long (1992); De Long et al. (1994); Fuhrman et al. (1992;1993); Hot springs: Barns et al. (1994); Ward et al. (1990); Soil: Liesack and Stackebrandt (1992); Stackebrandt et al. (1993); Bioleach environment: Goebel and Stackebrandt (1993; 1994).

1. Methodological problems exclude the possibility to quantify taxa determined to be present in clone libraries. These problems refer, among other reasons, to the extraction of nucleic acids, PCR-primer selectivity and sensitivity, cloning steps, and the dependence of the amount of PCR amplificates from undeterminable genomic properties, e.g. genome size and the number of rrn operons (Farelly et al., 1995). Thus, it is also not possible to decide whether the identified clones represent a majority or a minority population of the prokaryotic community. Nothing can be said reliably about species richness and species abundance.

2. Some sequences obtained from samples of widely separated locations may show surprising high similarities. This finding is based mainly on results obtained with samples taken from the Atlantic and Pacific but is probably also true for soil habitats.

3. Organisms cultured only rarely may be found in a great phylogenetic variety and almost ubiquitously distributed in nature, e.g., planctomycetes

(Ward, et al. 1995; Bond et al., 1995; De Long, pers. communication; Ogram, pers. communication)

4. The vast majority of the retrieved sequences are not identical to those of cultured strains. An exception refers to some marine cyanobacteria and to acidophilic strains from a bioleach environment.

5. As a consequence, no conclusions can be drawn about the physiological and biochemical properties of the unknown organisms, and their ecological role can not be elucidated.

6. While most bacterial sequences can be assigned to known phyla, archaeal sequences have been retrieved from marine (including Antarctic) environments that point towards the occurrence of completely novel kingdoms.

7. The occurrence of highly similar sequences is difficult to interpret. Do they reflect microheterogeneity of different rDNA operons or do they represent highly related strains?

8. Sequences of strains isolated from the same locations from which the DNA has been retrieved for the generation of a clone library are rarely identical to the clone sequences, nor to those available from the 16S rDNA database. The exception refers to the bioleach reactor study.

9. The molecular approach is only one, but an important step in the elucidation of ecological activities in natural samples. These studies must be accompanied by (i) the identification of cells through *in situ* probing, (ii) determination of cell activities through application of "functional" probes, (iii) determination of the metabolically active part of the population through analysis of mRNA, and (iv) attempts to isolate the as yet uncultured strains.

5. Identification of Symbionts

The possibility of identifying prokaryotic species directly in their natural habitat permits investigation of the taxonomic position and the identity of uncultured symbionts and parasites. Early 16S rRNA cataloguing studies on *Prochloron*, a symbiont of didimnid ascidians, had been successful because the symbiont occurred as an almost pure culture within the extracellular matrix of the host. The situation is much more complicated when either the symbiont occurs in small numbers only or when symbionts from different taxa are present within the same host. In this case, a similar strategy to that pointed out above, must be applied, e.g., isolation of rDNA, gene amplification, cloning and sequence analysis.

Symbioses are now attractive subjects for molecular microbial ecology because they comprise a small number of partners and because of the growing awareness of the importance of symbiotic relationships, i.e. the widespread

ability of microorganisms to colonize eukaryotes, and to perform biochemical functions. For example, work on anaerobic ciliates containing methanogenic archaeal endosymbionts (Embley et al., 1992a,b; Esteban et al., 1993; Finlay et al., 1993) has demonstrated that the ability to colonize anaerobic ciliates is common among methanogens. Symbionts originate from all but one (*Methanococcales*) of the major lineages of methanogens, and the symbioses appear to have formed independently from each other. The physiological basis of the association is thought to be interspecies hydrogen transfer from host organelles to methanogens (Finlay, 1990). There is currently no evidence for cospeciation of host and symbiont as closely related symbionts occupy hosts which are widely separated in the ciliate phylogenetic tree (Embley, pers. communication), and closely related hosts, such as *Metopus contortus* and *M. palaeformis* contain symbionts from different orders of methanogens.

Evidence for independent infections of aphids and their sister taxon, the mealy bugs, originate from the finding that the aphids' symbionts (*Buchnera*) evolved from the gamma proteobacterial ancestors (Munson et al., 1991a,b) while the mealy bugs symbionts are all drawn from a subgroup of the beta subdivision of proteobacteria (Munson et al., 1992). In contrast, the endosymbionts responsible for parthenogenesis or cytoplasmic incompatibility in different insects have been found to originate from a single lineage in the alpha proteobacteria (O'Neil et al., 1992; Stouthamer et al., 1993).

The symbionts of marine invertebrates are often chemoautotrophic symbiotic bacteria. Analysis of the chemoautotrophic symbionts from six marine invertebrates from diverse environments indicated that each host contained a single type of symbiont originating from a limited domain in the gamma-proteobacteria (Distel et al., 1988). The identity of cellulose degrading and nitrogen fixing symbiontia in the gills of ship worms (Distel et al., 1991) was investigated by rRNA sequence analysis and subsequent use of fluorescent probes; it could also be shown that the same (or closely related) species of the bacterium has a worldwide distribution in wood boring shipworms from different genera (Distel et al., 1991).

6. Detection and Quantification of Microorganisms in the Environment Using Probes Directed Against Ribosomal RNA

Once the composition of a community has been analyzed it is essential to identify which lineages are abundant in the environment. As outlined above, analysis of the composition of the clone library is not an appropriate approach. The method of choice used presently is the application of diagnostic oligonucleotide probes designed to bind to rRNA (Stahl et al., 1988). As

ribosomal RNA is a chimeric structure of conserved, semi-conserved and variable regions it is often possible to design probes of required specificity to detect taxa at various levels. The probes can be used to determine the relative abundance of rRNA from a particular organism or group in total environmental rRNA extracts (Stahl et al., 1988; Giovanonni et al., 1990). Amann et al. (1995) have published a comprehensive list of probe sequences for taxa above the species level which have been proven successful in the determination of organisms in the environment. Most probes have been developed to 16S rRNA but the larger and more variable 23S rRNA is attracting increasing attention (Manz et al., 1992).

Rather than using probes which are labeled either radioactively or carrying biotin or digoxigenin (Zarda et al., 1991), a more promising development is the use of fluorescent probes which can be applied to target rRNA within fixed whole cells (Amann et al., 1990a; DeLong et al., 1989; Manz et al., 1993; Stahl and Amann, 1991). This technique allows more precise quantification of individual microbes as cells can be counted directly and, if sampling permits it, observation of spatial distributions, even in mixed populations (Amann et al., 1992; Spring et al., 1992). However, the application of fluorescent probes to identify and count free-living microorganisms in more complex samples such as soil and water is in its early stages (Amann et al., 1990b, 1992; Devereux et al., 1992; Hicks et al., 1992; Manz et al., 1993) and more baseline studies are necessary to optimize this technique.

One application in which the use of fluorescent probes is mandatory is the description of those uncultured prokaryotic species for which the genomic uniqueness has been demonstrated but for which phenotypic properties are not determinable but required for the description according to the International Code of Nomenclature of Bacteria (Murray and Schleifer, 1994). Recently, the International Committee on Systematic Bacteriology has recommended to "implement the category "Candidatus" to record the properties of putative taxa of prokaryotes (Murray and Stackebrandt, 1995). The "Candidatus" is not a rank but a status without being formally in the Code of Nomenclature. A number of cases were listed which would be inadequate for formal naming of organisms for which the following characteristics are available only:

(i) When information provided exclusively by the analysis of clones generated from DNA that has been isolated directly from a natural sample.

(ii) When the authenticity of the genetic material is verified in the environment by re-amplification of DNA using sequence-specific PCR primers but the presence of cells containing that DNA has not been confirmed by microscopy or isolation.

(iii) When the origin of the genetic material from a living cell has been detected within the natural sample but the authenticity of the bacterial cell within the host is not proven by *in situ* hybridization.

(iv) When the origin of the genetic material from a living cell has been detected within the natural sample by *in situ* hybridization but information on properties other than its phylogenetic position and morphology is lacking.

The status of "Candidatus" is applicable for those uncultured prokaryotic cells for which not only the relatedness has been determined, but also the authenticity has been verified by *in situ* probing or a similar technique for cell identification, and structural peculiarities and certain metabolic and physiological features have been identified.

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