

## **MICROBIAL DIVERSITY AND THE MODERN APPROACH TO THE INVESTIGATION OF COMPLEX MICROBIAL ECOSYSTEMS**

G. AVGUŠTIN<sup>a)</sup>

<sup>a)</sup> Univ. of Ljubljana, Biotechnical Fac., Zootechnical Dept., Groblje 3, SI-1230 Domžale, Slovenia, Ass.Prof.,  
Ph.D., M.Sc., B.Sc.Biol.

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### **ABSTRACT**

Our knowledge about microbial diversity has been rapidly accumulating due to the application of the modern molecular biology based techniques. DNA sequence based taxonomy and phylogenetic studies were among the first that implemented molecular biology techniques but the field of microbial diversity seems to be the one that will profit most. Traditional estimations of the numbers of different microorganisms inhabiting our planet have been surpassed by the first molecular screenings of the natural microbial ecosystems and while everybody agrees that the known microorganisms can only represent a minor share of planetary microbiota, nobody can really estimate the actual size of it. Techniques that are not tied to the isolation and cultivation procedures are the only ones that could make progress in the field. A review of the history of implementation of molecular biology methods in microbiology is presented.

Key words: microbiology / microbial diversity / molecular biology / phylogenetics

## **MIKROBNA RAZNOLIKOST IN MODERNI PRISTOPI K PROUČEVANJU KOMPLEKSNIH MIKROBNIH EKOSISTEMOV**

### **IZVLEČEK**

Znanja o mikrobni raznolikosti se hitro kopičijo zaradi uvajanja modernih, na molekularni biologiji osnovanih metod. Na DNK sekvencah temelječa taksonomija in filogenetika sta bili prvi vidi, ki sta izkoristili molekularno biološke tehnike, zdi pa se, da bo prav področje mikrobne raznolikosti tisto, ki bo pridobilo največ. Prvi pregledi naravnih mikrobni ekosistemov z molekularnimi metodami so močno preseglji dotedanje zastarele ocene o številu vseh mikrobni vrst, ki naseljujejo naš planet. Medtem ko se večina strinja, da lahko doslej znani in opisani mikroorganizmi predstavljajo le droben delček svetovne mikrobiote, še nihče ne zna oceniti njene prave velikosti. Tehnike, ki niso pogojene in vezane na izolacijske in gojitvene postopke, so edine, ki obetajo napredek v tem osupljivem področju znanosti. Predstavljamo pregled uporabe molekularno bioloških tehnik v mikrobiologiji.

Ključne besede: mikrobiologija / mikrobna raznolikost / molekularna biologija / filogenetika

### Microbial diversity and the 16s rRNA

As biodiversity in general becomes one of the most important topics defining our views on the planet Earth and life on it, microbial biodiversity seems to have developed to a whole new level in the last couple of years. The introduction of molecular biology based methods and the breath taking development of computational facilities have enabled microbial taxonomists, evolutionists, and ecologists to study microbiology related problems in a completely new manner with an entirely different emphasis. The suggestions about the large discrepancies between the known and estimated biological species made in early 1990's on the basis of comparative studies may have been a surprise for many, but, due to the application of modern molecular biology based investigations in recent years, we now understand, that the estimates were, if anything optimistic, when considering the number of known microorganisms (Table 1). The statement that approximately 4, 12 and 5 % of all viruses, bacteria and fungi are already known, can be nothing but a grouse underestimation of actual microbial diversity. Especially if we bear in mind that many of the complex microbial ecosystems, like for example the animal gut, have at that time not been an object of any detailed molecular phylogenetic study. The investigations of such ecosystems performed in this very moment show however, that our fears (or hopes) were correct, expecting to find an abundance of previously unknown microorganisms when using molecular biology methods for describing microbial diversity (Amann *et al.*, 1995, Wilson *et al.*, 1996, Ramšak *et al.*, 1997, Wood *et al.*, 1998). It is now also widely accepted that we are not able to isolate and grow *in vitro* a large proportion of the microorganisms that inhabit a range of microbial ecosystems (Amann *et al.* 1995).

Table 1. Known and estimated numbers of microbial species and of some "higher" organisms (modified after Bull *et al.*, 1992)  
 Preglednica 1. Ocenjeno število vseh mikrobnih vrst in vrst nekaterih "višjih organizmov" in število danes poznanih vrst (povzeto po Bullu in sod. 1992)

Group	Known species	Estimated total species	Percentage of known species
Viruses	5,000	130,000	4
Bacteria	4,760	40,000	12
Fungi	69,000	1,500,000	5
Algae	40,000	60,000	67
Protozoa	30,800	100,000	31
Insects	800,000	6 – 10,000,000	8 – 13
Fish	19,000	21,000	90
Birds	9,198		~ 100
Mammals	4,170		~ 100

The 16S rRNA approach defined by Woese and colleagues in the 1980's and later developed by other research groups (Woese 1987, Olsen *et al.*, 1986, Olsen and Woese, 1993) has now already been applied to a fair number of cultured bacteria, and we can find over 10,000 sequences of small ribosomal subunits in the nucleic acids data banks, the majority also being covered by the Ribosomal Database Project (Maidak *et al.*, 1996). The underlying assumption of the 16S rRNA approach is that the sequential structure of the molecule is the authentic record of the evolutionary events of the molecule (i.e. gene) and the organism at the same time (Woese, 1987). The required conditions for such a molecule to be truly an authentic representative of its bearing organism, like the ancient nature, ubiquity, lack of the selection pressure, and of the

lateral spread, are all met by the small ribosomal subunit. Additionally, the molecule has a manageable length and a series of conserved and variable regions that allow a detailed analysis for a wide range of relationships (Fig.1).

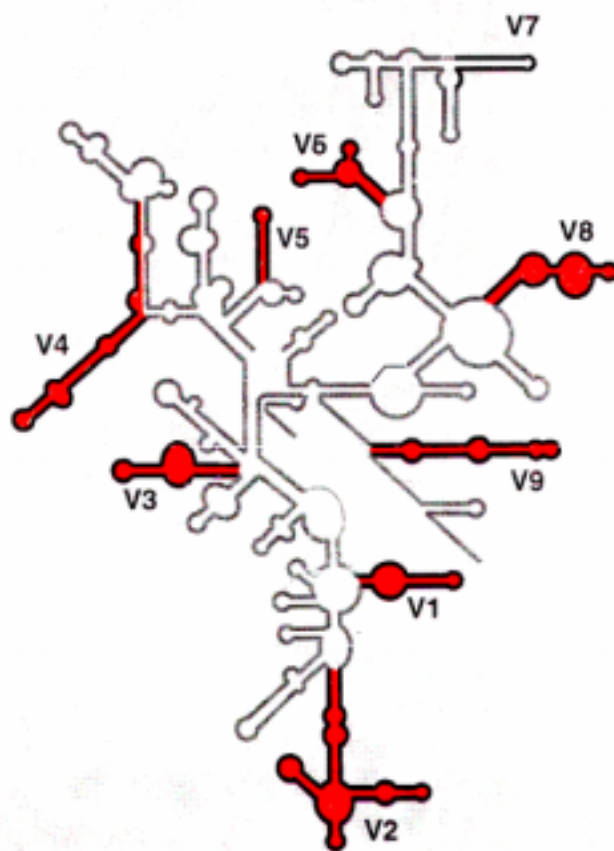


Figure 1. A 16S rRNA molecule (*E.coli*) showing nine variable regions.

Slika 1. 16S rRNA molekula (*E.coli*) in devet variabilnih regij.

A growing proportion of these sequences belongs to until now not cultivated microorganisms. The sequences were obtained mainly by PCR amplification of 16S rRNA genes from total community DNA from a variety of ecosystems (Stahl *et al.*, 1985, 1988, Liesack and Stackebrandt, 1992, Stackebrandt *et al.*, 1993, Manz *et al.*, 1996, Whitford *et al.*, 1998).

### Oligonucleotide probes developed from ribosomal sequences

The ribosomal sequences have been used not only for phylogenetic and taxonomic studies but also as a basis for oligonucleotide probe constructions. These can be used either in PCR detection systems as primers or in *in vitro* or *in situ* hybridization experiments as labelled oligonucleotide probes. Especially the later ones can be further exploited as a tool for direct detection and analysis of uncultured microorganisms by introducing fluorescently labelled probes into the individual microbial cells (Amann *et al.*, 1990, 1995, Tepšič and Avguštin, 1997). Using such a technique one can avoid the phenomenon called “the great plate count anomaly” (Staley and Konopka, 1985), describing the discrepancy between the direct microscopic counts and viable-cell counts, the former exceeding the later by several orders of magnitude. Such observations were published already in the late 70’s from investigations of

oligotrophic and mesotrophic aquatic habitats (Kogure *et al.*, 1979, 1980, Staley and Konopka, 1985), sediments (Jones, 1977) and soil (Torsvik *et al.*, 1990). Space distribution and even physiological status of individual microbial cells can be studied via epifluorescent microscopy using specific oligonucleotide probes (DeLong *et al.*, 1989, Wallner *et al.*, 1993). A number of previously unknown or unculturable bacteria, many among them true symbionts, were observed using the above described procedures. Symbiotic bacteria and archaea from chemoautotrophic invertebrates, insects and protozoa were investigated as well as pathogenic microorganisms, magnetotactic bacteria, bacteria composing marine picoplankton, microbial biofilms, and soil (for review see Amann *et al.*, 1995) and gut microbiota (Avguštin *et al.*, 1994, Whitford *et al.*, 1988).

### Quantitative methods for enumeration of targeted microorganisms in complex ecosystems

An upgrading of the system seems to be required, however, if the enumeration of specific microbial targets is to be performed. Laborious microscoping is one of the obstacles, and the homogeneous distribution of targeted cells on the microscope slide another, preventing efficient and accurate counting of labelled targets in a complex sample (personal experience). Automated image analysis facility enhancing the epifluorescent microscopy is therefore required or a different approach using the same oligonucleotide probe concept but a different sample treatment and signal detection is needed. Flow cytometry, designed for detection of fluorescently labelled microbial cells, offers an unprecedented way of analysing an enormous number of individual cells in a very short time (up to 7.000 cells per second) and seems to be the ideal tool when complex microbial samples are to be analysed or ecosystems monitored (for review see Davey and Kell, 1996). Indeed, such approach has been successfully used even for analysis of so complex microbial ecosystems as wastewater systems and the microbial communities within the activated sludge (Wallner *et al.*, 1995) and the rumen (unpublished results) (Fig 2.).

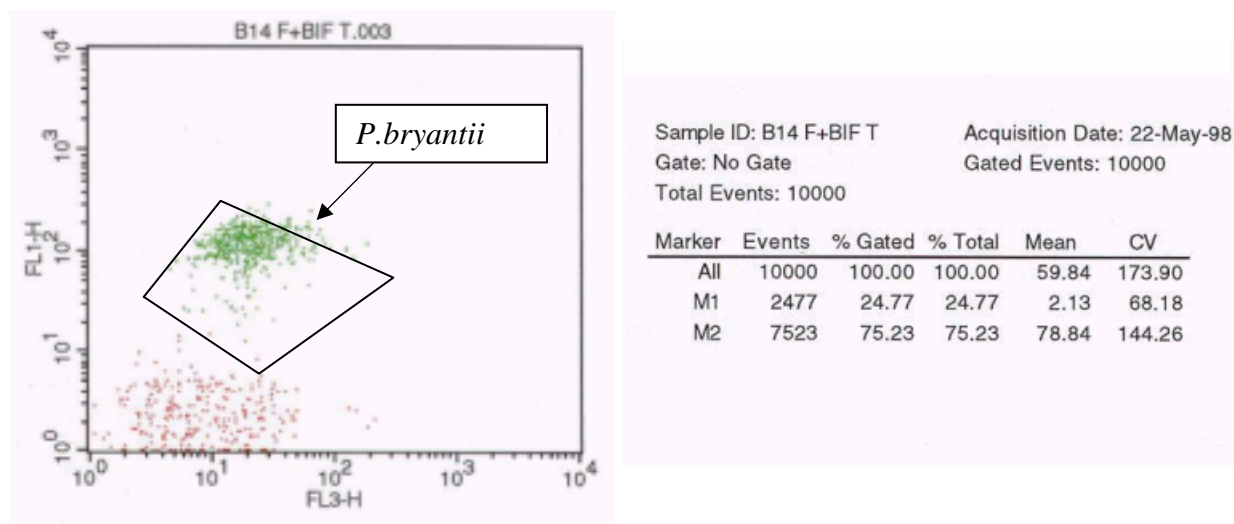


Figure 2. Flow cytometric analysis of a mixture of rumen bacteria *P.bryantii* B<sub>14</sub> and rat gut bacteria *Bifidobacterium* spp., hybridized with a B<sub>14</sub> specific oligonucleotide probe, labelled with FITC.

Slika 2. Pretočno citometrijska analiza mešanice vampnih bakterij *P.bryantii* B<sub>14</sub> in bifidobakterij iz črevesa podgan, hibridiziranih z B<sub>14</sub> specifično oligonukleotidno sondo, označeno s FITC barvilom.

Flow cytometry can offer even more i.e. the possibility of cell sorting, which can be exploited for further molecular analyses of uncultivated microorganisms (Wallner *et al.*, 1997).

### **Pitfalls of the molecular approach**

With growing amount of knowledge obtained from molecular studies of cultured and uncultured microorganisms new questions and even obstacles are encountered. We know little of the dynamics and the events taking place within individual PCR reactions and consequently of the biases as a result of the former. The numbers of ribosomal operons within individual genomes are known only for few better known bacterial species and even less is known about the sequence heterogeneity of the genes from different operons in the same organism. It has been reported however that within certain species this heterogeneity can reach 10 or more percents and little is known about the influence of the these sequence heterogeneities on the phylogenetic placements of microorganisms (Clayton *et al.*, 1995). The sample collection, cell lysis, nucleic acid extraction, PCR amplification, separation of amplified DNA and the sequencing and data analysis, each of the mentioned steps can be a source of errors or ambiguities, and little is known about the influence of any of them (for review see Wintzengerode *et al.*, 1997). The chimera formation is for example a phenomenon that deserves our attention having obviously the capacity of distorting the observed genotypic variability of a certain microbial community in a significant manner (Wang and Wang, 1996).

### **The future promises**

It seems that the sequencing of ribosomal genes from cultured microorganisms and establishing their taxonomic positions and phylogenetic relationships has already surpassed the peak of the activity. The analysis of the microbial biodiversity on our planet, however, has only started and the described approaches promise great progress in this field that has been so difficult to study until recently. Even more, with the detailed analysis of microbial endosymbionts in certain higher organisms, and the recent discovery of the parallel evolutionary development of the host and the microbial symbionts (Baumann *et al.*, 1995), we can actually expect new insights into the evolution and the development of microbial life on our planet. The fast progress that has been brought into the field through the use of molecular biology methods doesn't seem to be slowing down, also due to immense developments generated from the human genome project and computer industry in general.

### **POVZETEK**

Molekularna taksonomija in filogenetika sta bili prvi vеди, ki sta izkoristili in uspešno uporabili molekularno biološke tehnike. Danes smo priča hitremu kopičenju podatkov o mikrobni raznolikosti, ki temeljijo na DNK sekvencah. Zdi pa se, da bo prav področje mikrobne raznolikosti tisto, ki bo pridobilo največ. Prvi pregledi naravnih mikrobni ekosistemov z molekularnimi metodami so precej presegle dotedanje zastarele ocene o številu vseh mikrobni vrst, ki naseljujejo naš planet. Medtem ko se večina strinja, da lahko doslej znani in opisani mikroorganizmi predstavljajo le droben delček svetovne mikrobiote, še nihče ne zna oceniti njene prave velikosti. Tehnike, ki niso pogojene in vezane na izolacijske in gojitvene postopke, so edine, ki obetajo napredek v tem osupljivem področju znanosti. Največji napredek so raziskovalci dosegli z natančnim proučevanjem genov, ki kodirajo manjšo ribosomsko podenoto, t.j. 16S rRNK molekulo. Danes poznamo sekvenco že več tisoč genov za 16S rRNK

najrazličnejših mikroorganizmov, tudi takšnih, ki jih doslej še nihče ni izoliral in jih tudi ne znamo gojiti v *in vitro* pogojih. Na podlagi teh sekvenc pa lahko proučujemo ne le pravi, naravni ali t.i. filogenetski taksonomski sistem, temveč lahko sekvenčne podatke izkoristimo tudi za izdelovanje oligonukleotidnih sond. Z njimi lahko z *in situ* hibridizacijskimi poskusi specifično prepoznavamo tarčne mikroorganizme v kompleksnih vzorcih, jih prostorsko sledimo in spremljamo njihove fiziološke aktivnosti. S pomočjo pretočne citometrije pa lahko posamezne mikroorganizme celo izoliramo za nadaljnje molekularne ali imunološke preiskave. Predstavljamo pregled uporabe molekularno bioloških tehnik v mikrobiologiji, ter možnih težav in napak, ki se pojavljajo predvsem pri proučevanju naravnih ekosistemov.

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