Approaches for diversity analysis of cultivable and non-cultivable bacteria in real soil

P. Štursa[1], O. Uhlík[1,2], V. Kurzawová[1], J. Koubek[1], M. Ionescu[1], M. Strohalm[1,3], P. Lovecká[1], T. Macek[2], M. Macková[1,2]

[1]*Department of Biochemistry and Microbiology, Institute of Chemical Technology in Prague, Prague, Czech Republic*
[2]*Institute of Organic Chemistry and Biochemistry CAS, Prague, Czech Republic*
[3]*Laboratory of Molecular Structure Characterization, Institute of Microbiology CAS, Prague, Czech Republic*

ABSTRACT

Until recently, investigators had no idea how accurately cultivated microorganisms represented the overall microbial diversity. The cultivation-dependent approach is limited by the fact that the overwhelming majority of microorganisms present in soil cannot be cultivated under laboratory conditions. The development of molecular phylogenetics has recently enabled characterization of naturally occurring microbial biota without cultivation. There is a vast amount of information held within the genomes of cultivable and non-cultivable microorganisms, and new methods based on analysis of DNA allow to investigate this potential. In this work we show some aspects, advantages and disadvantages of classical and new approaches in taxonomical and functional description of bacteria present in natural microbial assemblages on the example of cultivable bacteria isolated from rhizosphere of plants, tobacco and black nightshade, planted in PCB contaminated soil. Biochemical analysis of isolates showed 8 different bacterial species. This identification was compared by discrimination using MALDI-TOF mass spectrometry and identity evaluation after sequencing of 16S rDNA. Six strains from original number of 8 were positively identified after 16S rDNA sequencing and their phylogenetic relations were compared. These analyses confirmed closed relations of all species (two of isolates exhibited the same characteristics and were discriminated as the same species *Pseudomonas stutzeri*) and also of *Burkholderia xenovorans* LB 400, a well-known PCB degrader. Nevertheless, only two isolates gave a positive reaction after amplification of the biphenyl dioxygenase gene and exhibited potential to degrade PCB. These results indicate that only a subset of the recovered molecular information, derived from active population based on molecular and functional analysis is relevant to microbial ecology.

Keywords: microbial diversity; cultivable bacteria; non-cultivable bacteria; SIP; DGGE; TGGE; T-RFLP; MALDI-TOF mass spectrometry

Microbiology, from its inception, battled with a difficult and often incorrect identification of newly discovered bacteria. Long time, only techniques based on isolation of microbial cells from natural samples and their cultivation at laboratory conditions, further biochemical or immunochemical characterization represented the only way for categorization of microbes. From present point of view all these methods are inaccurate and inconclusive.

In recent decades, a number of biological methods based on characterization of microbial DNA or RNA have been developed for reasons of better characterization. These methods are used to identify bacteria and describe bacterial DNA diversity in the case of individual bacteria, or DNA of entire microbial communities. In practice, the use of molecular biological methods includes isolation of the total DNA, amplification and analysis of 16S rRNA genes to get material for further analysis (Ryslava et al. 2003, Leigh et al. 2006). To characterize diversity of a given microbial community, different types of gradient electrophoresis, analysis of restriction fragment length polymorphism, or other methods can be used (de Cárcer et al. 2007).

Beside the methods characterizing microbial DNA, there are also other techniques based on analysis of characteristics and mostly highly conservative properties. From those, MALDI-TOF mass

spectrometry or analysis of the fatty acid methyl esters composition can be used. Powerful technologies that accurately identify organisms to species level combine with affordable pricing to provide the best choice for routine microbiology.

All of these methods provide very accurate results in the description of microbial diversity. However, major disadvantage of these methods is the inability to detect so-called non-cultivable (non-culturable) microorganisms representing 90–99.9% of the estimated amount of microorganisms on the planet (Amann et al. 1995, Hall 2007). Crucial change in this area posses the possibility to isolate DNA or RNA straight from the environmental sample and to use molecular biology techniques for characterization of the whole communities or individual strains with respect to the attempts of the concrete research goal. This solution is the only one in the cases of non-cultivable microbes, and the new approach – studying the whole communities – is called metagenomics. DNA-based molecular techniques permit the comprehensive determination of microbial diversity but generally do not reveal the relationship between the identity and the function of microorganisms. The first direct molecular technique to enable the linkage of phylogeny with function is DNA-based stable isotope probing (DNA-SIP). Applying this method first helped to describe the utilization of a wide variety of compounds, including various xenobiotics (Radajewski et al. 2003, Uhlík et al. 2008).

Biochemical and immunochemical tests

Biochemical and immunochemical tests represent one of the oldest methods used for microbial identification and characterization. Many commercial kits exist, which allow determination of biochemical properties of individual pure cultures on the basis of microbial phenotypes (Busse et al. 1996). In culture-based detection methods a preliminary step to amplify the microbial population is included. Enrichment is the most common, while highly unpopular, technique used to accomplish the amplification just described. The targeted bacterium is then isolated from the enrichment medium using selective and differential media, and identified on the basis of multiple biochemical properties. Culture media can be nonselective or selective. Nonselective media allow a wide variety of bacteria to grow (e.g., nutrient agar or plate count agar etc.). Selective media allow only certain organisms to grow because they have specific inhibitors added to the media (e.g., the bile salts in MacConkey agar). Generally, these methods of bacterial identification suffer from two major drawbacks. First, they can be used only for organisms that can be cultivated *in vitro*. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species. Another disadvantage is that most of these techniques are manual and require significant effort and time to be performed.

Alternatively, further identification is performed by immunochemical or genetic techniques. However, an additional drawback other than the long response time, is the requirement for special chemical consumables that add considerably to the logistic burden and costs. These can increase operational costs by hundreds of dollars per hour.

Mass spectrometry

Mass spectrometers provide an accurate measurement of the molecular weight of a compound in addition to information on the fragmentation of the molecule that helps deduce its molecular structure. A key principle of this method is different mobility of current carriers with different molecular weight at electromagnetic field (Lay 2000, Ruelle et al. 2004). With the implementation of MALDI (Matrix-assisted Laser Desorption/Ionization) in combination with TOF (time of flight) detector type, great progress of mass spectrometry occurred in biochemistry and microbiology for example as a phenotypisation method for identification of prokaryotes. The ionized molecules are thought to be primarily proteins or fragments of proteins, but may also include higher molecular weight fragments of lipid and oligosaccharide components of the cell wall. In the last few years, it has become popular as a tool for typisation or identification of bacteria by analyzing whole cells instead of extracted protein fragments. The greatest advantage of the whole-cell technique is quick preparation, about 10 min, and relatively low sample size (Krishnamurthy et al. 1996). Subsequent evaluation of the spectrum may not be so clear. Mass spectrum can be affected by a variety of factors, whose control is necessary for the emergence of replicable result. It was found, that the range depends in part on the age of the culture, the time lag between inoculation and analysis, the type of device, a laser-type matrix, and chemicals used for the partial lyses of the cells, and even on treatment before analysis. The sensitivity of the model depends on the quality of

the data and the nature of the species, but studies so far have indicated > 95% success.

In general, it can be said that the use of MALDI-TOF MS has a great potential in environmental microbiology where microorganisms represent diverse physiological and phylogenetic groups. Its advantages over other techniques include minimal sample preparation, rapid results, and very low reagent costs. The disadvantage is a higher purchase price of instrumental equipment, evaluation software and database.

Electrophoresis in temperature and denaturing gradient

Molecular strategies based on PCR, cloning, sequencing, and probing enabled biologists to examine the total bacterial community in a sample without any a priori knowledge of the species present in the mixture (Amann et al. 1995, Ercolini 2004).

Bacterial diversity in environmental samples is usually determined by the characterization of isolated strains. Isolated bacteria may account only for a minor portion of the total bacterial diversity originally present in the sample. A new approach in microbial ecology is based on the analysis of bacterial genetic information which can be performed without cultivation. This approach has greatly enhanced the ability to assess bacterial diversity in ecosystems such as ground and surface water environments (Niemi et al. 2001). After isolation of the total bacterial DNA, variable regions of the 16S rRNA gene are amplified by PCR (Torsvik et al. 1998). The similar sized PCR-products are separated by subsequent denaturating or temperature gradient gel electrophoresis (DGGE or TGGE) and the resulting diversity patterns are analyzed and compared. The principle of these two very similar methods is the very low rate of partially denatured double stranded DNA (dsDNA) migration. The special primers with a GC clamp are used for the PCR. The GC section becomes a part of DNA, which allows only partial denaturation of the sample in the gel. A main difference itself is the electrophoresis, which takes place under gradually changing conditions – in DGGE in a denaturing gradient of urea, in TGGE in the temperature gradient. Depending on the sequence differences the strands stop at different points in the gel, resulting in the separation of the DNA. In the TTGE technique, dsDNA is subjected to an increasing time-dependent temperature gradient and melts again depending on the sequence loaded in the acrylamide gel. Partial melting of the DNA reduces its mobility allowing the differentiation of diverse sequences (melting is sequence-specific) on the basis of the positions achieved in the gel. Proper phylogenetic identification of microorganisms is very problematic. Of course, rDNA of known strains can be used and it is possible to compare the retention indexes, but excision of the proper band from the gel, DNA extraction and the sequencing (Torsvik et al. 1998, Muyzer 1999) can give information about community and particular species. Limitation for this method is again the inability to use large parts of DNA. Sequences up to 500 bp are usually used.

Restriction Fragment Length Polymorphism Analysis (RFLP)

Restriction Fragment Length Polymorphism describes differences in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination. Products, made by PCR reactions, are split by suitable endonuclease and fragments are divided in electrophoresis. Microorganism can be classified according to the number and size of obtained fragments. Major disadvantage of this method is the need of only one type of DNA (cannot be used for the analysis of the whole community).

RFLP is still a technique used in marker-assisted selection. Terminal Restriction Fragment Length Polymorphism (TRFLP or sometimes T-RFLP) is a molecular biology technique initially developed for characterizing bacterial communities (community fingerprinting method) in mixed-species samples. TRFLP works with PCR amplification of DNA using a forward (or reverse) primer that has been labeled with a fluorescent tag. The PCR products are then digested using restriction enzymes and the resulting patterns visualized using a DNA sequencer. The results are analyzed either by simply counting and comparing peaks in the TRFLP profile (Osborn et al. 2000), or by matching peaks from one or more TRFLP runs to a database of known species.

DNA sequencing

DNA sequencing is the most accurate and reliable terminal procedure for identification of most micro-

organisms. This application is ideal for situations where a rapid and accurate identification is mandatory. Microbial identification based on sequence data of the 16S rRNA gene, which is universally found in all bacteria and is considered as one of the best target genes (evolution chronometre) for use in new species identification. Sanger's principle of sequencing is premature termination polymer-modified response to a radioactive or fluorescent labelling. The biggest disadvantage is the inability to sequence fragments greater than 1000 bp; it is caused by the deficiencies in the distribution of higher molecular weight fragments. Other disadvantage is, of course, the high price of DNA analysis and requirements for the high purity of DNA (Hall 2007). New sequencing technologies such as pyrosequencing (so called 454 technology) have been successfully used as rapid and efficient tools to enable in-depth analysis of bacterial composition and diversity of consortia of environmental microbes (Kling 2003).

Stable isotope probing (SIP)

The fundamental questions in environmental microbiology are as pertinent as ever: How individual microbial species are involved in the biodegradation of a pollutant (Tillmann et al. 2005), and which functions are attributable to which microorganisms in the natural environment (Radajewski et al. 2003). Several recently published methods have adopted the converse approach to address these questions, by first establishing the biological process and then identifying the microorganisms that are involved. An example is the technique of stable isotope probing (SIP); a term coined to describe the use of ^{13}C-labelled growth substrates to link microbial function with identity via selective recovery of 'heavy' ^{13}C-labelled DNA.

The method involves introducing a stable isotope-labelled substrate into a microbial community and following the fate of the substrate by extracting diagnostic molecular markers, such as fatty acids and nucleic acids (Manefield et al. 2004, Uhlík et al. 2008) from the community. These molecules provide identifying information about the organism that incorporated the substrate (Wacket 2004). Stable isotope probing allows direct observations of substrate assimilation in minimally disturbed communities, and thus represents an exciting new tool for linking microbial identity and function. The use of isotope ^{13}C is predominant, the use of ^{15}N is less common (Uhlík et al. 2008). The most important feature of DNA-SIP is that the heavy DNA (^{13}C-DNA) fraction collected following extraction and density-gradient centrifugation contains the combined genomes of a microbial population that is able to incorporate the labeled substrate into their biomass. Therefore, 'universal' polymerase chain reaction (PCR) primers that are used to amplify the SSU rRNA genes of *Bacteria*, *Archaea* or *Eukarya* are integral for the identification of microorganisms involved in the process of interest. As any method, the SIP has its limitations and pitfalls. One of the basic problems is the requirement for sufficient amount of isotope-labeled atoms in the molecules studied (Radajewski et al. 2003). Another problem is a relatively high price of the labeled substrates.

RESULTS AND DISCUSSION

Examples of experimental approaches used for characterization and identification of environmentally important strains.

In our experiments we cultivated two different plant species, black nightshade and tobacco, in soil long-term contaminated with PCB. After 6 months of plant cultivation in PCB contaminated soil the effect of presence of plants and spectra of bacterial species were evaluated. Culturable bacteria present in the rhizosphere of plants were isolated and identified using different types of classification and identification approaches.

The following section shows examples of microbial analysis in PCB contaminated soil based on different culturable and nonculturable approaches and comparison of their results showing microbial diversity in rhizosphere of two plant species and bulk soil. Primarily, our interest was focused on rhizosphere Gram-negative PCB-degrading species. These bacteria were first selected by enrichment cultivation on biphenyl as a sole carbon source, and then by PCR identification of the gene bphA (for biphenyl dioxygenase) in DNA of selected bacteria (Ryslava et al. 2003, Barriault and Sylvestre 2004).

Biochemical test NEFERMtest 24 and analysis of 16S rDNA genes

All isolated culturable bacteria were first extracted by pyrophosphate and pure individual species were biochemically identified. NEFERMtest 24 commercial kit (Lachema a.s.) is used for routine

Table 1. Summary of identified bacterial strains using biochemical kit NEFERMtest 24

	1	2	3
Ochrobactrum anthropi	●	●	●
Burkholderia mallei	●	●	–
Burkholderia cepacia	●	●	–
Chryseomonas luteola	●	●	–
Pseudomonas fluorescens	–	●	–
Agrobacterium radiobacter	●	–	–
Oligella ureolyitca	●	●	–
Stenotrophomonas maltophilia	●	–	–

● presence of bacterial strain; – absence of bacterial strain. Pots were vegetated with two different plants: 1 – vegetated with black nightshade; 2 – vegetated with tobacco; 3 – control nonvegetated soil. After finishing of the experiment bacteria were extracted from soil by pyrophosphate and isolated on PCA (plate count agar) medium. Individual pure colonies grown on agar medium after 24 h were biochemically characterized by NEFERMtest 24. Eight different species were taxonomically classified

characterization and identification of Gram-negative non-fermenting bacteria. This group contains many environmentally important strains participating in geochemical cycles of elements, or degrading different types of xenobiotics. The test allows identifying the representatives of cytochrome oxidase positive, gram-negative rods. Identification is based on a set of 24 biochemical tests, and positive or negative response is evaluated to give numerical code typical for a certain species. The results of NEFERMtest24 are shown in Table 1.

From the results in Table 1 it is evident that rhizosphere contains several culturable species not detected in bulk soil. Several bacteria were identified in rhizosphere of both plant species, nevertheless, there were also species typical only for one type of plant root area.

Section of 16S rDNA genes is conserved part of ribosomal DNA, and analysis of the primary sequence is used for taxonomic identification of bacteria. In our study, only two obtained isolates were positively tested for the presence of biphenyl dioxygenase gene. Their 16S rRNA genes were amplified using specific primers and then sequenced. The strains were classified as *Pseudomonas plecoglossicida* originally identified with NEFERMtest24 as *P. fluorescens*, and *Ochrobactrum anthropi* identified also with NEFERMtest24.

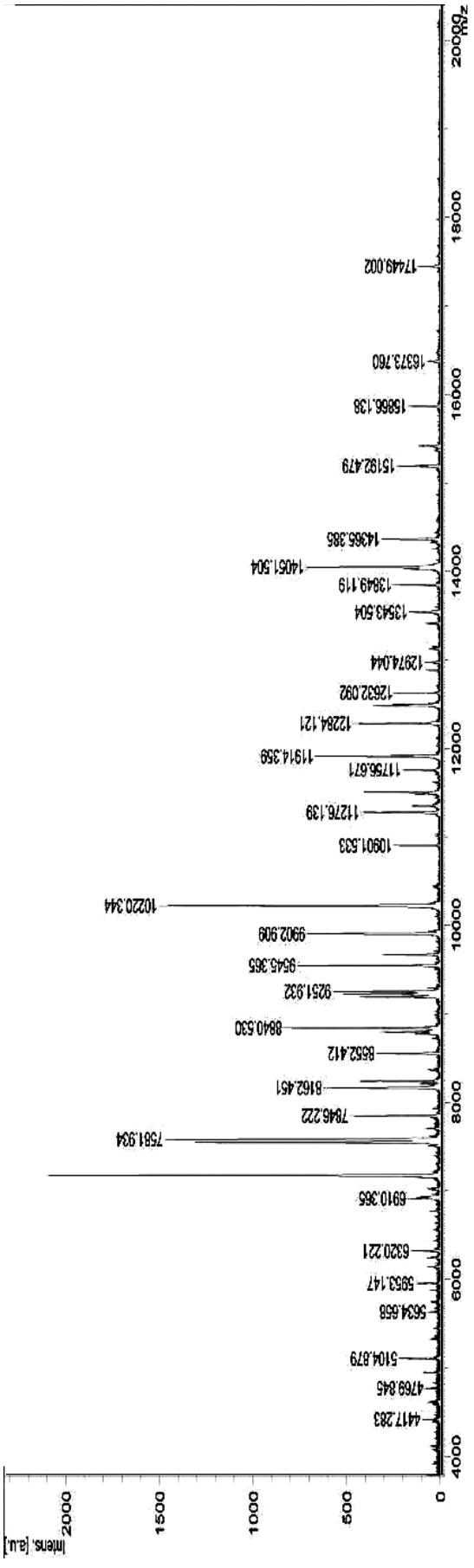

Figure 1. Whole cell MALDI-TOF profile of chosen bacterial isolate *P. plecoglossicida*. Peaks represent different, mostly ribosomal proteins

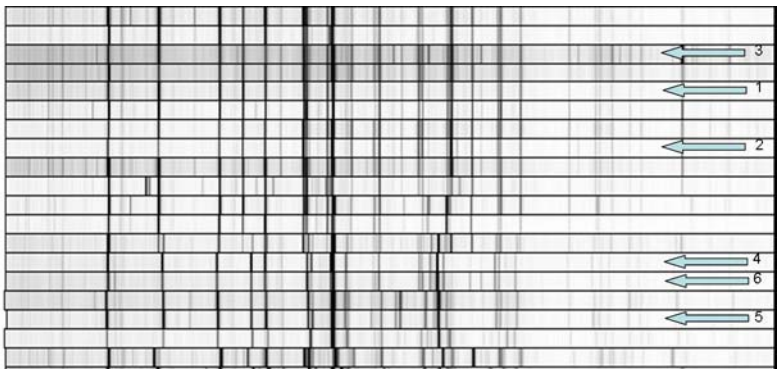

Figure 2. Graphical expression of the MALDI-TOF spectra profiles of various isolates. Six isolates were identified by sequencing of 16SrDNA and the level of similarity in MS spectra profiles were compared with the gene similarity of 16SrDNA (see also Figure 3)

1 – *Pseudomonas putida*; 2 – *P. putida*; 3 – *P. plecoglossicida*; 4 – *P. stutzeri*; 5 – *P. alcaligenes*; 6 – *P. stutzeri*

MALDI-TOF Mass spectrometry

Other relatively new approach which can be used for fast differentiation of isolated culturable bacteria is MALDI-TOF mass spectrometry (van Baar 2000, Lay 2001). The method is preferably useful when many different pure cultures are isolated from environmental samples and we need to unify them to the same species or at least related groups prior to identification. The classification of bacteria can be done only if appropriate database of spectra is available.

Procedure is fast and very simple – the sample of cells (1 µl) is applied to metal plate and covered with the layer of 3,5-dimethoxy-4-hydroxycinnamic acid in mixture of acetonitril and 5% trifluoroacetic acid 70:30 (v/v). The samples are then analyzed using a mass spectrometer Biflex IV. The machine is externally calibrated with standards containing peptides or proteins of known molecular weights. The example of such a spectrum is shown in Figure 1.

Example of the profiles constructed from spectra of MS analysis of individual bacterial isolates cultivated and isolated from soil consortia is shown in Figure 2. The strains 1–6 exhibited the same or very similar spectra and they were identified by 16S rDNA analysis. It can be assumed that these bacteria are identical or closely related bacterial strains. Phylogenetic tree of these bacteria is shown in Figure 3.

T-RFLP (Terminal Restriction Fragment Length Polymorphism)

This method was used to analyze microbial diversity in samples of contaminated soil vegetated with black nightshade and tobacco plants. The experiment was based on an analysis of the total DNA which was isolated from the soil. Purified DNA samples were PCR-amplified using 8F and 926R primers targeting eubacterial 16S rRNA genes. 8F primer was labelled with 6-carboxyfluorescein (6 FAM) on the 5'end. Amplified DNA was digested with HhaI restriction enzyme (NEB, 10 000 U/ml) following the manufacturer's instructions. Sequence analysis by capillary electrophoresis was performed commercially. Spectra obtained by capillary electrophoresis (Figure 4) showed much higher number

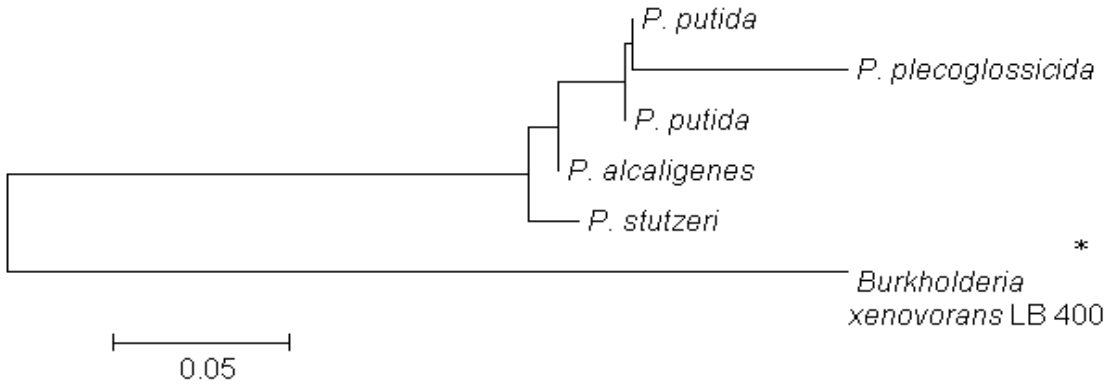

Figure 3. Example of phylogenetic tree, made with the Mega 4 software. * The strain *Burkholderia xenovorans* LB 400 (reference strain) (Sylvestre and Barriault 2004) shows phylogenetic distance from isolated bacteria

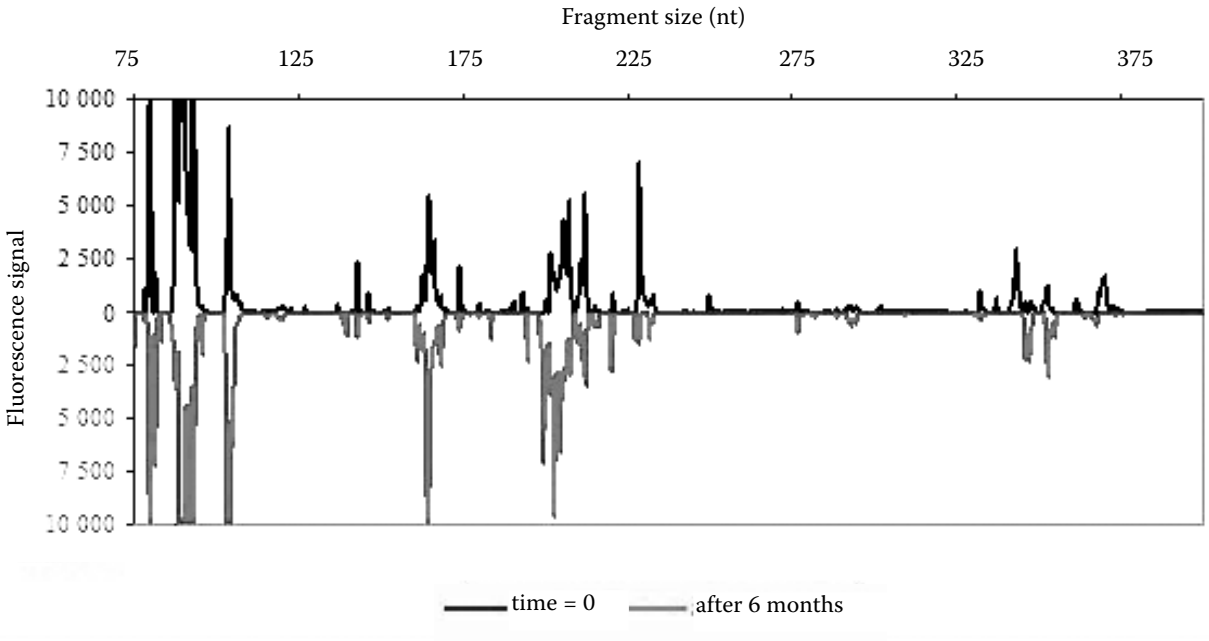

Figure 4. Example of T-RFLP profiles of 16S rRNA gene amplicons digested with HhaI. The first figure is a spectrum of microbial diversity at the beginning of the experiment. The second figure is a spectrum of microbial diversity at the end of the experiment (6 months)

of bacterial species in rhizosphere samples than was previously found by cultivation (in spectrum each peak represents one bacterial species). Further sequencing would show also the species present.

Identifying microorganisms responsible for recognized environmental processes remains a great challenge in contemporary microbial ecology. Traditionally, it has been difficult to attribute a recognized microbially-mediated process to the organism(s) responsible for that process *in situ*. Direct retrieval of rRNA sequences can be used to detect specific rRNA sequences of uncultured bacteria in natural samples and their taxonomical classification. In the analysis of bacterial flora constituted by plural microorganisms, gene analysis methods such as clone library, DGGE (Denaturing Gradient Gel Electrophoresis), and T-RFLP methods can be employed. Of them, the T-RFLP method is particularly useful for the rapid analysis of samples containing unknown bacterial species or many bacterial species. Only in the last few years methodological innovations have provided access to the relationship between the function of a microbial community and the phylogeny of the organisms accountable for it. Among the limited number of elegant methodologies capable of identifying microorganisms responsible for particular biogeochemical processes, stable-isotope probing (SIP) holds considerable promise.

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Corresponding author:

Prof. Dr. Ing. Martina Macková, Vysoká škola chemicko-technologická v Praze, Fakulta potravinářské a biochemické technologie, Technická 5, 166 28 Praha 6, Česká Republika
phone: + 420 220 443 021, e-mail: Martina.Mackova@vscht.cz