METHODS OF STUDYING DIVERSITY OF BACTERIAL COMMUNITIES: A REVIEW*

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Bacterial life is a predominant component of all environments, occurring in communities usually of complex diversity. Bacteria are engines of globally important processes which makes them subject of interest to many scientific studies. Although many kinds of methods have been developed and microbial ecology has undergone a profound change in the last two decades with regard to methods employed, the analysis of community composition and function still remains a great challenge. In this article we present an overview of methods commonly used for the study of bacterial diversity. Emphasis was placed on cultivation, biochemical and chemotaxonomic, and molecular-genetic based methods.

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INTRODUCTION

Bacteria belonging to prokaryotes are ubiquitous and essential components of the Earth's biota. They catalyse unique and indispensable transformations in the biochemical cycles of biosphere, produce important components of Earth's atmosphere, and represent a large portion of life's genetic diversity. They are also producers of important compounds that serve as a nutrient source, usable by all parts of the food chain. The numbers of prokaryotes on the Earth are estimated to be 4–6 × 10^{30} cells. Most of them occur in the open ocean, in soil, and in ocean and terrestrial subsurface (Whitman et al., 1998). Another very important microbial ecological niche is animal body. It has been estimated that the microbes in human bodies collectively make up to 100 trillion cells, 10-fold the number of human cells, and suggested that they encode 100-fold more unique genes that our own genome. The majority of these microbes reside in the gut, have a profound influence on human physiology and nutrition, and are crucial for human life (Qin et al., 2010). Because of the immense importance of bacteria, there is a need for more detailed and predictive understanding of the bacterial communities responsible for activities mentioned above and how they may respond to environmental stress and changes (Hirsch et al., 2010).

The late 19th century is considered as the beginning of bacterial taxonomy and Ferdinand Cohn in 1872 was the first who classified six genera of bacteria mainly based on their morphology. At that time

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growth requirements and pathogenic potential were the most important taxonomic markers, besides morphology. Many of the pathogenic bacteria known today were discovered just between 1880 and 1900. At the beginning of the 20th century physiological and biochemical features started to be used, in addition to morphology, as important markers for classification and identification of microorganisms. Later enzymes have been studied and metabolic pathways have been described. Since the 60s of the 20th century microorganisms have been also identified and classified based on chemotaxonomy (composition of cell constituents) and genotype (Schleifer, 2009). In the 80s of the 20th century scientists have found the discrepancy between counts of cells visible under the microscope and the equivalent total viable counts after the cultivation. This phenomenon, so-called 'great plate count anomaly', was the first indication that majority of bacteria and archaea on Earth remain 'unculturable'or 'as yet uncultivated' on artificial media *in vitro*. This fact has contributed to the development of molecular biological methods to study microbial communities, without the need of cultivation (Vartoukian et al., 2010; Uhlík et al., 2013). In nature less than 1% of bacteria can be cultured with currently available methods. Relative proportion of bacteria growing on agar plates (colony forming units – CFU, determined as a percentage of culturable bacteria in comparison with total cell amount) vary from 0.1 to 1% in pristine forest soils and to 10% in environments like arable soils (Torsvik et al., 1998; Cottrell, Kirchman, 2000). Amann et al. (1995) reported culturability of bacteria in water 0.001–3%, activated sludge 1–15%, sediments 0.25%, and in soil 0.3%. Studies performed in the 1970s using anaerobic culture-based techniques identified more than 400–500 distinct bacterial species in the human gut, but approximately 60–80% of gut microbes simply cannot be grown by conventional *in vitro* techniques (Dave et al., 2012). Despite the fact that cultivated microorganisms represent only a minor part of microbial diversity in nature, cultivation is essential for isolating live bacterial cells in pure culture and determining their metabolic pathways. Therefore cultivation is still one of the key techniques used in microbiology (Uhlík et al., 2013).

Most of information on bacterial diversity concerns genetic and taxonomic diversity, but to understand the role of communities in different environments it is essential to have knowledge of both community and functional diversity. Therefore, there are various approaches to study the microorganisms at the community level. In principal, these methods can be divided according to different criteria. In this article we divided them into cultivation, biochemical and chemotaxonomic, and molecular-based methods. Another criterion for grading methods can be for example taxonomic level because each technique is suitable for identification at various taxonomic levels.

Cultivation methods

Cultivation methods are based on inoculation and incubation of growth media and have been used for more than 100 years to detect microorganisms. These techniques provide at least two benefits: amplification of microbial material and purification of single organisms along with their direct descendants, which allows their further characterization. The composition of culture media depends on the aims of study. Elective media with rich sources of nutrients are used to obtain the widest possible spectrum of bacteria from environmental samples (Mandal et al., 2011). On the contrary, for enumerating and isolating a certain group of microorganisms media consisting of specific nutrients composition, energy sources and selective antimicrobial agents are required (Vlková et al., 2015). Since 1990 a range of chromogenic media have been developed that are designed to target microorganisms with high specificity, without the need of subsequent identification. Such media exploit enzyme substrates that release coloured dyes upon hydrolysis, thus resulting in target microorganisms forming coloured colonies that can easily be differentiated from other bacteria species. Ideally, other bacteria should either be inhibited completely by selective agents or form colourless colonies thus allowing target bacteria to 'stand out' against background flora (Perry, Freydière, 2007). Currently conventional bacterial testing methods relying on selective and chromogenic media are the most commonly used for detection and identification of indicator and pathogenic microorganisms in food and clinical microbiology (Relman, 1998; Mandal et al., 2011). Serial enrichment of microorganisms in the presence of various nutrient and energy sources, analyses of their macromolecular composition and their metabolic by-products, and the use of specific immunologic reagents have created a variety of systems for microbial classification and identification (Relman, 1998). These methods are very sensitive, inexpensive, except chromogenic media, which are invariability more expensive than conventional media and can give both qualitative and quantitative information on the number and the nature of microorganisms present in a sample. On the other hand, these methods are time consuming. They require several days to give results, because they rely on the ability of the organisms to multiply to visible colonies. Moreover, culture medium preparation, inoculation of plates, and colony counting makes these methods labour intensive. Limitations of cultivation methods may be due to heterogeneity of samples matrices, physical form or different viscosity owing to the content of fats and oils. Moreover, bacteria are not uniformly distributed in environment or in samples. Another limitations include the difficulty in dislodging bacteria or spores from soil particles or biofilms, incomplete selectivity of culture medium, growth conditions, and different

Table 1. Advantages and limitations of the cultivation method

Method	Advantages	Limitations
Cultivation	sensitive	‘unculturable’ and ‘as yet uncultivated’ bacteria not detected
	inexpensive	dislodging bacteria from sample
	amplifies microbial material	selectivity of culture medium
	qualitative and quantitative information	different growth rate
		slow

growth rate of microorganisms (Kirk et al., 2004; Mandal et al., 2011), see Table 1.

Biochemical and chemotaxonomic methods

Sole carbon source utilization profiles. The principal of this technique is based on the ability of bacteria to utilize different substrates under various conditions. Microorganisms are cultivated in microtiter plates containing different carbon sources and indicator in wells. In the case that substrate is metabolized the degradation is accompanied by colour change. Methods of sole carbon utilization profile are generally used for characterization (detection of metabolic pathways and enzymes) and strain identification of pure cultures, but also allow studying functional aspects at community level. The first aspect is microbial ‘community function’, which implies actual expressed catabolic activity. In contrast, ‘functional diversity’ indicates its potential activity, i.e. the capability of the community to adapt metabolism (catabolism) and/or the relative composition and size of constituent populations to varying abiotic conditions (microclimate and added substrates). Information on both functional aspects is essential (Preston-Mafham et al., 2002). Currently, various types of biochemical kits are commercially available. For most of them the inoculation of pure culture into wells is required. Systems that may determine metabolic potential of whole microbial communities in the studied environment are also available (Stefanowicz, 2006).

The first who studied microbial communities by biochemical systems (on the basis of patterns of community-level sole-carbon-source utilization) were Garland and Mills (Garland, Mills, 1991). They used commercially available microplates developed by BIOLOG, Inc., designed for bacterial isolates identification, which allow simultaneous testing of 95 separate carbon sources. Portions of whole environmental samples instead of a single organism were inoculated into the wells. Utilization of substrates by bacteria was determined by the colour change of tetrazolium violet after the reduction by NADH produced by the microorganism during the reaction. The intensities of colour changes were determined spectrophotometrically. The substrate utilization rate between different groups of microorganisms is varied, therefore high variability in velocity and density of colour development can be observed, depending on microbial community composition. The microbial communities are consequently characterized by their metabolic fingerprints. Currently, special plates designed for ecological study of whole microbial communities are produced, e.g. Eco plates (Biolog, Inc.) containing 31 different substrates in triplicate, which allows statistical analysis (Baudoin et al., 2001; Stefanowitz, 2006; Button et al., 2015). This method is applied especially for evaluation of relative differences between functional diversity (the role of individual components) of microorganisms, for example in plant rhizospheres or communities in sites contaminated by industrial pollution, it does not allow identification of species composition in a sample. Limitations of metabolic profiling are that the method selects microorganisms capable of growing under the experimental conditions, favours fast growing microorganisms, is sensitive to inoculum density, and reflects the potential, and not *in situ*, metabolic diversity. Moreover, the carbon sources used for the tests may not correspond to those present in the sample (Kirk et al., 2004), see Table 2.

Fatty acid analysis. Fatty acids, integral part of membrane lipids, are essential components of all living cells. They have a greatly diversified structure and simultaneously are highly biospecific, therefore are used as biomarkers for studying microorganisms. Microbial fatty acid analysis may serve for both identification and taxonomic classification of pure cultures, and studying microorganisms in environmental samples or laboratory cultivated bacterial mixtures (Zelles, 1999).

A technique widely used for bacteria identification is the fatty acid methyl ester analysis (FAME), which is based on a four-step procedure: extraction of fatty acids from cellular lipids, methylation of fatty acids, extraction of FAMEs, and their analyzation by gas chromatography (GC). Cellular lipids are present in viable or non-viable cells, so extraction of fatty acids is not restricted to living organisms. In environmental studies the fact that in complex samples both cellular and extracellular lipids are found must be taken into consideration. Extracellular lipids may exist in a stable form in the sample organic matter, so results do not provide information about actual diversity but rather insight into the history. In addition, extracellular lipids

Table 2. Advantages and limitations of biochemical and chemotaxonomic methods

Method	Advantages	Limitations
Sole carbon source utilization profiles	fast relatively inexpensive differentiates between communities	selects culturable fraction of community favours fast growing organisms sensitive for inoculum density reflects the potential, not *in situ* metabolic diversity tested carbon sources may not correspond to those present in sample does not allow identification of species composition
Fatty acid analysis	culture independent detects viable cells, or both live and dead qualitative and quantitative information sensitive to changes in community structure identifies specific organisms	identification restricted to culturable bacteria fatty acids derived from eukaryotic organisms not all species can be detected not all species can be distinguished from complex profile
Protein analysis	generates large amount of data informs about gene functions identifies specific organisms links functions with identity detects changes under varying conditions informs on post-translational modification	technologically challenging demanding

can be derived not only from bacteria but also from eukaryotic microorganisms and macroorganisms, e.g. plants (Haack et al., 1994; Zelles, 1999).

A similar approach is the analysis of the phospholipid fatty acid (PLFA) pattern of bacteria. In the first phase all cellular lipids from a sample are extracted by suitable solvents and buffer, and subsequently divided into fractions: neutral lipids, glycolipids, and phospholipids (polar lipids). Methyl esters of phospholipid fatty acids, prepared by mild alkaline methanolysis, are analysed by gas chromatography or mass spectrometry (GC/MS). Phospholipids are an essential constituent of all cellular membranes and are used as a marker for microbial community description because their derived fatty acids significantly differ between individual bacterial groups. Whereas phospholipids rapidly degrade after cell death and do not occur in storage components, the method provides information about the actual microbial composition. The FAME analysis, in comparison with PLFA, is more rapid and less demanding than PLFA, on the other hand provides less reliable results (Makula, 1978; Gattinger et al., 2003). Both methods mentioned above are suitable for studying bacteria and eukaryotic microorganisms, in which fatty acids are connected by ester bonds. Archeal polar lipids contain fatty acids connected by ether bonds, thus they are not hydrolysed using the standard PLFA protocol. Polar lipids in Archea (so-called phospholipid ether lipids) have a unique structure, and can be easily distinguished from bacterial and eukaryotic phospholipids. They are composed of di- and tetraethers of glycerol or more complex polyols with side chains consisting of isoprenoids, which can only be liberated after ether cleavage with a strong acid such as hydriodic acid (Gattinger et al., 2003).

A method based on cellular lipids analysis is also the analysis of isoprenoid quinones, which represent an important group of isoprenoid lipids occurring in the cytoplasmic membrane of most prokaryotes. The isoprenoid quinones analysis is based on quinones extraction by organic (nonpolar) solvents, purification, and analyzation using various chromatographic methods, UV spectrophotometry, or mass spectrometry. This method is applicable to all environmental samples from which an absolute amount of microbial biomass $\geq 10^9$ cells can be collected, and in combination with molecular methods it should provide accurate and reliable information about population dynamics and community structure (Collins, Jones, 1981; Hiraishi, 1999; Schleifer, 2009). Isoprenoid quinones play an important role in electron transport, oxidative phosphorylation and, possibly, active transport, and thus they constitute a part of both respiratory and photosynthetic system of microorganisms. Two major structural groups of bacterial isoprenoid quinones can be recognized: naphthoquinones and benzoquinones. For classification not only the type of quinone, but also length and saturation of polyprenyl side chain is significant.

In addition, for microbial community description other fatty acid analysis based methods have been used, including the analysis of muramic acid, teichoic acid components, or lipopolysaccharides fatty acids charac-

teristic for Gram-negative bacteria and cyanobacteria (Gehron et al., 1984; Vestal, White, 1989).

Fatty acid profiles of pure cultures are compared to each other or to an established database to assist in the polyphasic taxonomic description of these organisms. The community structure is interpreted based on the database of pure cultures peripherally, unless there is a unique lipid which can serve as a true biomarker for given microbial strains (Zelles, 1999; Hopkins et al., 2001). Information about the community composition is based on the frequency and ratio between individual fatty acids, which form relatively constant portion of cell biomass. So, changes in fatty acids profiles represent changes in microbial population (Kirk et al., 2004). Even though fatty acids analysis represents a culture independent technique, determination of the community composition by software is restricted only to reference fatty acid profiles from individual organisms grown on the culture medium. Further, not all bacterial species can be detected. Disadvantageous is also the fact that some bacterial species are not sufficiently characteristic in respect to fatty acids composition, and thus cannot be distinguished from the whole community profile (Haack et al., 1994; Zelles, 1999).

Protein analysis. Another way how to study bacteria is to examine the protein content. The proteins found in bacteria provide not only an indirect genetic information on the organism, but are also found in a great abundance, which in turn should lead to a high detection sensitivity. The type of proteins present in bacterial cells is immense and their amounts vary greatly. Some proteins are unique to specific bacteria and may serve as biomarkers. The ribosome is an organelle found in all cells that coordinate protein synthesis, therefore ribosomal proteins have been proposed as suitable biomarkers for chemotaxonomic characterization of bacteria (Teramoto et al., 2007).

Complex information about the protein composition provides the field of proteomics, which is traditionally defined as the analysis of a complete set of proteins (proteome) of a given cell or organisms at a given time under specific conditions. The term metaproteome reflects the compound proteome of whole microbiota found in the environment, i.e. mixed microbial communities (Wilmes, Bond, 2004). Metaproteomics is widely accepted as a key technique in the postgenomic era to investigate global protein synthesis and gene expression. In this context, the large-scale study of proteins expressed by indigenous microbial communities should provide information to gain insight into the functional dimension of the environmental genomic dataset and help achieve a major goal of environmental microbiology: the ability to link individual bacterial species to function (Cash, 2000; Wilmes, Bond, 2006). More specifically, analyses of metaproteome allow tracking new functional genes and metabolic pathways, and identifying proteins preferentially associated with specific stress (Maron et al., 2007). The metaproteome analysis implies the development of different technical steps, from the extraction of proteins from the environmental matrix to the resolution of their diversity and identification. For an exhaustive recovery of proteins (cellular + extracellular), bacteria may be lysed either directly in the environmental matrix before extraction, purification, quantification, and analysis or by an indirect strategy, in which proteins are extracted, purified, and separated from organisms that have been previously extracted from the environmental matrix. The most crucial step in metaproteomics is the protein pool extraction. The complexity of indigenous microbial communities, the heterogeneity of natural environments, and the presence of interfering compounds make the extraction difficult (Wilmes, Bond, 2006). Once the protein samples are obtained, different biochemical methods can be applied for metaproteome analyses according to the type of information and the level of required results. In general, the combination of separation techniques (electrophoresis, chromatography) and proteins identification by mass spectrometry (MS) provides the best possible results. The traditional way to separate proteins in a complex mixture is by the use of two-dimensional (2D) gel electrophoresis, when proteins are separated according to net charge in the first dimension (isoelectric focusing, IEF) and by molecular weight in the second dimension (SDS-PAGE). The 2D electrophoresis has the ability to resolve a large number of proteins including those with post-translational modification as well as unique forms of proteins that result from differential mRNA splicing or proteolysis and provide ‘proteofingerprint", which can be analysed using computer software to identify changes in protein expression in the two samples. Subsequently spots of interest may be excised, digested by trypsin, and analysed. However chromatographic based techniques are often preferred as a more reliable tool for proteins separation (Wilmes, Bond, 2004; Maron et al., 2007). Although various types of mass analysers are used in microbial proteomics including tandem mass spectrometry (MS-MS), quadrupole mass spectrometers or ion trap mass spectrometers, a revolutionary progress in protein analysis has occurred with the onset of two critical ‘soft ionization' technologies, namely electrospray ionization (ESI) MS and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS (Graham et al., 2007). Especially MALDI-TOF MS is widely used not only in environmental analyses but for routine classification and identification of bacteria, based on characterization of ribosomal subunit proteins as biomarkers, in numerous fields, including the food industry and public health (Teramoto et al., 2007).

Proteomics provides qualitative data on the proteins encoded by the bacterial genomes together with quantitative data on the response of proteins synthesis

Table 3. Advantages and limitations of molecular-based methods

Method	Advantages	Limitations
Labelling	studies metabolically active fraction links functions with identity DNA, RNA, PLFAs or proteins can be targeted studies *in situ* sensitive	expensive labelled substrate sensitivity of DNA stable isotope probing interpretation of data
Nucleic acid hybridization and re-association	phylogenetic identification studies *in situ* DNA or RNA can be studied thousands of genes can be analyzed qualitative and quantitative information	technically demanding error prone comparative database cannot be built less sensitive not accurate in samples of complex diversity design of specific probes
Polymerase chain reaction (PCR)-based methods	amplifies specific RNA fragments *in vitro* sensitive phylogenetic identification qualitative and quantitative information studies gene expression	inhibition by co-extracted contaminants differential amplification formation of artefactual products formation of chimeric molecules formation of deletion and point mutants choice of primers

under defined environmental condition (C a s h , 2000). Using this approach to elucidate functional components of microbial ecosystems has huge potential for the study of environmental microbiology but still poses enormous challenges (W i l m e s , B o n d , 2006).

Molecular-genetic methods

The basic premise of molecular-genetic methods is that you are working on nucleic acids. DNA extracted from a sample represents the total metagenome, including components that are no longer viable, whereas RNA is synthesized only by active growing cells and degrades relatively rapidly. In prokaryotes, messenger RNA (mRNA) is usually very short-lived and indicates which genes are active at the time of extraction, but ribosomal RNA (rRNA) is more stable as it possesses secondary structure and is associated with ribosomal proteins, so in theory, it could survive for months in moribund or dead cells in environmental sample. However, in cells that are or recently have been active, there are many thousands of molecules of rRNA. Thus the analysis of rRNA abundance and diversity has been used to indicate the most active bacterial population, despite the fact that the number of ribosomes varies between groups. More precise information relevant to particular functions can be obtained from mRNA but it presents more technical difficulties. Both mRNA and rRNA can be converted to DNA using enzyme reverse transcriptase (RT) (H i r s c h et al., 2010). The best characterized molecules used for bacterial systematics are small subunit rRNA gene sequences (16S rRNA). These molecules are universally distributed in the cells, exhibit constancy of function, change in sequence very slowly, the number of mutations corresponds to the evolutionary distance between strains, moreover contain high conservative regions across the bacteria domain and simultaneously variable regions specific for a certain taxon. Additional advantage of 16S rRNA is that it could be easily isolated (F o x et al., 1980; U h l í k et al., 2013). The analysis of rRNA genes provides a framework for assigning sequences to genera and species, appropriate for investigating the microbial community diversity, but it cannot always resolve the species and provides insufficient discrimination at the sub-species level. For intra-species variation, protein-encoding genes that have higher levels sequence variation will permit differentiation of closely related individuals. An added advantage is that most of these genes occur with only a single copy per genome, and therefore give a more reliable indication of the relative abundance of different groups than the 16S rDNA (H i r s c h et al., 2010).

While microbiologists were previously limited by their inability to characterize uncultured organisms, the advent to so-called ‘cultivation independent’ methods has provided researchers with the ability to determine the composition of bacterial communities and identify numerically important, but not yet cultured organisms (F o r n e y et al., 2004). Molecular-genetic methods represent a culture-independent approach, which is characterized by high specificity and the detection limit is approximately 10^3 of cells/g or ml of sample, depending on a particular method (M a n d a l et al., 2011). They began to be applied since mid-1960s, when protein electrophoresis was firstly used in ecol-

ogy researches to detect genetic variation in samples of individuals from different populations and species (Baker, 2009). In microbiology these methods started to be used later, because techniques that were optimized for eukaryotic cells were not fully applicable for prokaryotes. Currently, molecular-genetic methods are commonly used for identification, determination of the phylogenetic position of unknown species, and also for studying the microbial community composition (Wintzingerode et al., 1997; Zhang et al., 2002). The advantages and limitations of selected techniques are listed in Table 3.

Labelling to link functions with identity. Stable isotope probing (SIP) can be considered as a transition between biochemical and molecular based methods that allow studying biochemical processes involving the participation of microorganisms in the natural environment without the need of their cultivation. SIP exploits physical properties of the atoms that constitute all cellular components, in particular the isotopes of carbon. Substrates labelled by stable "heavy" isotopes are added into the environmental sample, metabolized by a certain group of microorganisms, and incorporated into their cellular structures. For substrate probing stable isotopes (especially ^{15}N, ^{2}H, and most commonly ^{13}C) are used. Incorporating a labelled isotope into the cells considerably increases the differences of the density between heavy (labelled) and light (unlabelled) fractions, which can be separated from each other by equilibrium density-gradient centrifugation (ethidium bromide, cesium chloride, cesium trifluoroacetate). Even small differences in the isotope ratio can be measured by isotope-ratio mass spectrometry (IRMS) or by secondary ion mass spectrometry (SIMS). For identifying the bacteria involved in the labelled substrate degradation serve biomarkers such as DNA, RNA, PLFAs or proteins which can be analysed by a range of molecular and analytical techniques (Radajewski et al., 2003; Neufeld et al., 2007a; Lünsmann et al., 2016).

Phospholipid-derived fatty acid SIP (PLFA-SIP) is the method of choice when probing a population composed of relatively low cell number or growth rates and therefore incorporating minimal amount of labelled substrate. However utility of PLFA-SIP may be limited, because resolving profiles composed of multiple species can be problematic. PLFAs are analysed by a combination of gas chromatography and isotope ratio mass spectrometry (GC-c-IRMS) without prerequisite separation (Treonis et al., 2004; Neufeld et al., 2007b). More accurate identification results are provided by nucleic acids-based SIPs. RNA-SIP enables the analysis of 16S ribosomal RNA (rRNA) genes and DNA-SIP enables analysis of both 16S rRNA genes and physiological genes from target organisms. RNA-SIP is more rapid, almost 10-fold, because DNA-SIP experiments require cell division in the presence of labelled substrate to achieve sufficient incorporation for separation of labelled DNA, and also offers higher sensitivity than DNA-SIP. On the other hand, a sufficient amount of high-quality microbial rRNA for RNA-SIP is not extractable from all environmental samples. The advantage of DNA-SIP is a relatively easy DNA extraction and stability compared with rRNA (Radajewski et al., 2003; Winderl et al., 2010).

The second method, which has been developed to identify metabolically active bacterial groups in a sample, is 5-bromo-2′-deoxyuridine (BrdU) probing. BrdU is a thymidine nucleotide analogue that can be incorporated into the DNA of dividing cells. DNA extracted from cells that incorporated BrdU can be isolated by immunocapture and then compared, using any method appropriate for community DNA analysis, to the unlabelled DNA from the less active majority (Hirsch et al., 2010). Thanks to this method it is possible to determine the most active bacterial groups in environmental samples under various conditions. BrdU probing has been used to study microbial community changes, to detect microbes that grew in response to the availability of various carbon sources or in agricultural and bioremediation studies, both *in vitro* and *in vivo* (Scupham, 2007).

Nucleic acid hybridization and re-association. One of the most common techniques used in systematic studies, for locating homologous DNA sequences and measuring their overall base pair differences respectively, is DNA–DNA hybridization (DDH). The general principle of DNA–DNA hybridization requires shearing the DNA into small fragments (600–800 base pairs), dissociation of hydrogen bonds by high temperature to obtain single-stranded DNA, and subsequently re-association by decreasing temperature. DNA fragments of assayed organism and reference organisms are mixed, denatured, and re-associated to form a heterodublex (Bledsoe, Sheldon, 2009). Also total community DNA from environmental samples can be used to determine whether two samples share the same kind of organisms, regardless species composition knowledge (Theron, Cloete, 2000). Genetic relatedness between the bacterial strains is determined by quantitative measurement of the mutually connected nucleotide bases of DNA fragments after re-annealing (Ezaki et al., 1989). The specific pairings are between A-T and G-C, and the overall pairing of the nucleic acid fragments is dependent upon similar linear arrangements of these bases along the DNA (Rosselló-Mora, Amann, 2001). Wayne et al. (1987) reported that the phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA–DNA relatedness and with difference in melting temperature 5°C or less (also called the thermal denaturation midpoint, T_m), both values must be considered. However this best-known recommendation is not a strict standard, also more stringent DNA–DNA hybridization values

have been used. Although DNA–DNA hybridization was one of the few universally applicable techniques available that could offer truly genome-wide comparisons between organisms, it has several important drawbacks. Because relatively large quantities of DNA (in comparison with PCR-based techniques) of high quality are required, the whole process of performing DDHs often becomes rather time-consuming, labour intensive, and technically demanding. This method is also error prone and cannot be used to incrementally build up a comparative database, in contrast to sequence information. Due to great diversity of microbial populations this technique is not suitable for the study of complex environmental samples (Rosselló-Mora, Amann, 2001; Goris et al., 2007).

The gross genetical structure and diversity in bacterial communities have been assessed by DNA melting-profiles and re-association analyses. In these analyses, total prokaryotic DNA (mixture of DNA from different bacterial types that are present in different proportions) is extracted from environmental samples, thermally denatured, and then re-associated. The re-association of single stranded (melted) DNA is measured as a decrease in absorbance using a spectrophotometer. The hybridization and re-association rates depend on the sequence homology. Thus, as the microbial community diversity increases, the rate of re-association of DNA extracts from the community decreases. Under defined conditions the molar concentration of nucleotides in single stranded DNA at the beginning of the re-association and time in seconds needed for 50% re-association ($C_0t_{1/2}$) is proportional to the DNA complexity (heterogeneity) and can be used as a diversity index. DNA re-association can provide useful information on the overall diversity and changes in the community structure. However, higher resolution and more accurate information about gross genetical structure are provided by PCR-based methods (Torsvik et al., 1998).

The easiest way to detect specific nucleic acid sequences is through direct hybridization of a probe to bacterial nucleic acids. The hybridization techniques relying on the specific binding of single stranded nucleic acid probes are an important tool in molecular bacterial ecology (Theron, Cloete, 2000). Oligonucleotides (less than 20 nucleotides) or polynucleotides (more than 50 nucleotides) serve as the probes, designed from the known sequences of various specificity from domain to species specific probes. Species specific probes complement the most variable regions, while more general probes target more conserved regions of the molecule (Ezaki et al., 1989; Theron, Cloete, 2000). Hybridization can be done on extracted DNA, RNA or *in situ*. Early applications of *in situ* nucleic acid hybridization relied on the use of isotopically labelled oligonucleotides that bound to the RNAs and following autoradiography organisms could be identified, but currently fluorescent probes are often preferred. The method, known as fluorescent *in situ* hybridization or FISH, has been used successfully to study the spatial distribution of bacteria in various environmental samples. FISH is a staining technique that allows phylogenetic identification of bacteria on a single cell level in mixed assemblages without prior cultivation and also allows determine the cell morphology of uncultured bacteria. The principle of this technique is hybridization of specific fluorescent dye labelled rRNA-targeted oligonucleotide probes to the target sides and detection of hybridized cells by epifluorescence and confocal laser microscopy, or by flow cytometry. FISH with polynucleotide DNA probes and FISH with oligonucleotide probes targeted to mRNA have also been described. This technique is commonly used for both quantitative and qualitative determination of bacteria and for the study of spatial and temporal dynamics of individual bacterial populations in their habitat. However, identification of microorganisms from environmental samples of unknown composition can be limited because the design of specific probes is based on the sequence database which is not complete for all bacteria (Theron, Cloete, 2000; Pernthaler et al., 2001).

Base-pairing of complementary sequences by hybridization is also the principle of the DNA microarray technique. This method was first described in 1995 and it has the ability to simultaneously display the expression of thousands of genes at a time. Specific binding of DNA allows a target DNA or RNA to hybridize to a specific complementary DNA (cDNA) probes immobilized on the surface of array. Each probe is made of thousands of cDNAs or oligonucleotides, each specific for a gene, DNA sequence, or RNA sequence of interest. DNA microarray has a wide range of applications. Except microbial population studies it is currently being applied also for example in quality control, clinical diagnostics, biomedical research of cancer, or drug discovery and development (Hadidi et al., 2004).

Polymerase chain reaction (PCR)-based methods. Research in the fields of population and evolutionary biology has been revolutionized by the introduction of polymerase chain reaction (PCR). Using this technology, the researcher can *in vitro* amplify specific DNA fragments in a virtually unlimited quantity (more than 10^9 copies after 30 cycles of DNA synthesis) (Hirsch, 2010). PCR involves enzymatic synthesis of a particular DNA (or sometimes RNA) sequence. The DNA region to be amplified is determined by the base sequences of a pair of oligonucleotide primers, which are complementary to binding sites situated on either side of the target sequence. According to the selection of primers, it is possible to detect either functional genes or sequences specific for a certain group of bacteria (e.g. genus or species specific primers). These primers binding sites reside on opposite strands of the template DNA so that primers have their 3′ hydroxyl

ends oriented towards each other. DNA polymerase-mediated extension of each annealed primer therefore proceeds in the direction of the other primer (Birt, Baker, 2009). PCR is based on the three simple steps required for any DNA synthesis. Each cycle begins with denaturation of template DNA by high temperature (usually 94 or 95°C) into single strands. The temperature is then lowered to permit annealing of the primers to the appropriate sequences of each original strand (45–60°C). Finally the temperature is elevated to achieve optimal polymerase activity (70–72°C), and extend the anneal primers as directed by the template strands. The amount of PCR products increases geometrically. PCR products can be visualized by ultraviolet (UV) transilluminator after electrophoresis through agarose gel staining by an appropriate dye, they appear as a bright band of appropriate size, and then can be readily characterized using any of numerous techniques (Birt, Baker, 2009).

Due to the power of the PCR to amplify a small amount of DNA, organisms occurring in small number in an environment are now detectable. Also, the sample volume required for analysis is significantly reduced and micro-habitats are now open for investigation. PCR amplification has become the method of choice for obtaining rRNA sequence data from microbial communities or pure cultures. Full length 16S rDNA can be amplified either directly or after reverse transcription of rRNA with a set of primers binding to conserved regions of 16S rDNA/rRNA. Although it is a routine method for pure cultures, several problems arise when the methods are applied to environmental communities including inhibition of PCR amplification by co-extracted contaminants, differential amplification or formation of artefactual PCR product (Wintzingerode et al., 1997).

In microbial ecology, quantitative PCR (Q-PCR or real-time PCR) is now widely applied to quantify the abundance and expression of taxonomic and functional gene markers within the environment. Q-PCR-based analyses combine 'traditional' end-point detection PCR with fluorescent detection technologies to record the accumulation of amplicons in 'real time' during each cycle of the PCR amplification. By detection of amplicons during the early exponential phase of the PCR, this enables the quantification of gene (or transcript) numbers when these are proportional to the starting template concentration. When Q-PCR is coupled with a preceding reverse transcription reaction, it can be used to quantify gene expression (RT-Q-PCR) (Smith, Osborn, 2009). The random amplified polymorphic DNA (RAPD) is a common PCR-based DNA fingerprinting technique used in molecular ecology to determine taxonomic identity, assess kinship relationships, analyse mixed genome samples, and create specific probes. The amplification protocol differs from the standard PCR conditions in that only a single random oligonucleotide primer is employed and no prior knowledge of the genome subjected to analysis is required. The amplification products are resolved on agarose gels and polymorphisms serve as dominant genetic markers, band patterns are compared to determine percent similarity. Main advantages of the RAPD technology include suitability for work on anonymous genomes, applicability to problems where only limited quantities of DNA are available, efficiency, and low expense (Hadrys et al., 1992; Franklin et al., 1999). Restriction fragment length polymorphism (RFLP), also known as amplified ribosomal DNA restriction analysis (ARDRA), is another tool used to study microbial diversity that relies on DNA polymorphisms. In this way PCR-amplified rDNA is digested by cutting restriction enzyme. Different fragment lengths are detected using agarose or non-denaturing polyacrylamide gel electrophoresis. RFLP banding patterns can be used to screen clones, detecting structural changes in microbial communities or to distinguish the standard from mutated genes (Kirk et al., 2004). Terminal restriction fragment length polymorphism (T-RFLP) is a technique that addresses some of limitation of RFLP. It is based on the restriction endonuclease digestion of fluorescently end-labelled PCR products. Either one or both primers used in the PCR can be labelled. If both, each can be labelled with a different fluorescent dye. Upon analysis, including separation by gel or capillary electrophoresis with laser detection of the labelled fragments using an automated analyser, only the terminal, end-labelled fragments are detected. So this technique permits an automated quantification of the fluorescence signal intensities of individual terminal restriction fragments (T-RFs) in a given community fingerprint pattern (Osborn et al., 2000).

Besides the above mentioned techniques, there exist many other polymerase chain reaction-based techniques to estimate the microbial diversity and community composition, e.g. ribosomal intergenic spacer analysis (RISA) (Fisher, Triplet, 1999), single strand conformation polymorphism (SSCP) of DNA (Loisel et al., 2006), denaturing gradient gel electrophoresis (DGGE), and temperature gradient gel electrophoresis (TGGE) (Muyzer, Smalla, 1998).

CONCLUSION

There are various approaches to determinate the composition of microbial communities and their role in different environments. In addition to the classical tools mentioned in this article, modern methods such as next generation sequencing (NGS) have recently started to be applied. However due to its comprehensive character this field should be a subject of a separate review. Each of the commonly used techniques has its advantages and limitations. The selection of a suitable method depends especially on the aims of the

scientific research. However, combination of multiple techniques provides the best possible knowledge of the community diversity, structure, and function.

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