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Molecular ecology of microbes: A review of promises, pitfalls and true progress

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Abstract: Ecosystems, including engineered ones, are complex systems in which microorganisms occur in heterogeneous communities. Their behaviour in the environment is often unknown due to the lack of proper detection and identification techniques. Molecular ecology is a new field in which microbes can be recognized and their function can be understood at the DNA/RNA level without unreliable steps of cultivation of microbes. During the last few years genetically modified microbes have been constructed by recombinant DNA techniques for putative use in the environment. The slow progress in this field is due to the lack of integration of microbial ecology and molecular biology. In the present review, examples will be given of the use of DNA probes and marker genes in our study on the ecology of genetically modified microbes and wild-type recalcitrant microorganisms that are difficult to cultivate or even 'non-culturable'. Emphasis is given to the development and use of oligonucleotide probes directed towards 16S rRNA, to detect microbes in various engineered ecosystems: (i) *Frankia* in root nodules, and (ii) propionate-oxidizing sulfate-reducing bacteria in anaerobic granular sludge. Expression of genes is demonstrated by studies on the localization of *nifH* transcripts in root nodules of *Coriaria* and *Alnus*. In addition we will describe examples of the use of marker genes (*gusA* gene and *aphV* gene) to study competition and genetic stability of released engineered *Rhizobium* and *Streptomyces* strains.

Key words: Oligonucleotide probes; *Frankia*; *Streptomyces*; *Syntrophobacter*; In situ hybridization

Introduction

Molecular ecology

Microbial ecology has been dependent on the use of conventional microbiological methods for a long time. Isolation and cultivation of pure cultures on selective media or in situ measurements of microbial activity were the main parameters used in ecological research. Many microorganisms, including most of our model organisms, like e.g. the actinomycete *Frankia*, are difficult to

cultivate. This problem has been approached by characterizing the microbes on the DNA level, without unreliable cultivation steps. This new approach in ecology is defined as 'Molecular Ecology' and covers the range of applications of genetic and molecular techniques and theories in ecology.

What are engineered ecosystems?

Engineered ecosystems are man-made complex systems in which microorganisms live, like in natural ecosystems, in heterogeneous communities. Often the identity of the microbes in such

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environments is unknown, due to the lack of proper detection and identification techniques. During the last few years genetically modified microbes have been constructed by recombinant DNA techniques for potential use in the environment. Detection of these organisms requires the development of molecular techniques that often are identical to the methods to be used for detection of wild-type strains. In the present review, examples will be given of the application of DNA probes and marker genes in the research on the ecology of Genetically Modified (= 'Engineered') Micro-Organisms (GMMOs/GEMs) and recalcitrant or unculturable wild-type microorganisms. Emphasis is given on the development and use of oligonucleotide probes to detect microbes in various engineered ecosystems, like e.g. *Frankia* in root nodules, and propionate-oxidizing sulfate-reducing bacteria in anaerobic granular sludge. In addition we will describe examples of the use of marker genes (*gusA* gene and *aphV* gene) to study competition and genetic stability of released (engineered) *Rhizobium* and *Streptomyces* strains.

Uncultured versus unculturable

It is a well-known fact that many microorganisms do not grow under conditions routinely used in the laboratory. We simply do not know the growth conditions, or we do not know how we can reverse differentiation of the cells to return them into a viable stage. Heterocysts from filamentous cyanobacteria do not or only occasionally germinate and divide. These cells are dependent on the autotrophic, filamentous cells: they are fully alive, but non-culturable. Many microbial communities are extremely diverse due to complex interactions between organisms. More and more we have to realize that cultivation of microorganisms from the environment is a simplification of reality, leading to a wrong interpretation of the ecosystem. In order to prevent the discussion whether or not cells are indeed 'unculturable', we will use the term 'uncultured' here to define cells in their actual environment. Future investigations will learn us whether the cells are indeed unculturable.

Monitoring microbes

No ecosystem without biodiversity

Microbes are widespread in the environment and play an essential role in the synthesis and degradation of organic compounds. Although autotrophic, multifunctional microbes exist, such microbes seldomly dominate and have no strong competitive ability. Apparently ecosystems tend to develop increased biodiversity in which organisms form complex communication and interaction systems. Usually, microbial ecosystems are highly heterogeneous and consist of members with different, specialized metabolic capacities. In order to describe genetic heterogeneity, i.e. biodiversity, one needs reliable and accurate methods to detect and to identify these different bacterial groups.

Since the pioneering work of the founders of microbiology, Koch, Pasteur, Beijerinck and Winogradski, microbial ecology, in contrast to animal and plant ecology, was mainly dependent on measuring viable counts of microbes under laboratory conditions. Later it became clear that many microbes in nature occur in a dormant or starvation stage, from which they are difficult to revive. It also became clear that many microbes live in complex interaction with others, under conditions that are difficult to simulate in pure cultures. The drawbacks of conventional microbiological cultivation methods have received special attention after the observations of Colwell and co-workers on the occurrence of so-called 'non-culturable' *Escherichia coli* strains [1], as well as on the occurrence of a large number of unculturable human pathogens, e.g. *Aeromonas* [2], *Campylobacter* [3], *Legionella* [4], *Salmonella* [5], *Shigella* [6], and *Vibrio* [6]. These observations have had important implications for microbial ecology, particularly in the field of the ecology of pathogens [7,8].

The discussion about the reliability of microbiological detection and identification techniques has further been intensified due to the introduction of recombinant DNA technology in industrial and environmental biotechnology. Studies on the fate of released GEMs require the develop-

ment of sensitive and accurate detection techniques (see overviews in [9,10]). The introduction of recombinant DNA techniques and other molecular biological approaches in ecology have opened the new field of 'Molecular Microbial Ecology'. The application of new techniques, particularly the DNA probing technology, enables research in this field to describe ecological processes such as adaptation, selection and genetic variation on a molecular basis of nucleic acids, i.e. gene stability, gene transfer and gene expression.

During the last few years a number of review papers have appeared on survival of released GEMs in the environment [11–13] and an increasing number of proceedings and books have been focussed on this subject (see e.g. [9,10]). The present paper summarizes the major key points and pitfalls in the use of molecular techniques in microbial ecology, as exemplified by our own recent observations.

Why monitoring microbes in the environment?

Monitoring of microorganisms in the environment can be done to obtain a better understanding of a microbial ecosystem and the survival strategies of microorganisms [14,15], or more particularly to assess the risks of introduced wild-type isolates or GEMs in the environment. In the past, extensive work has been done to assess the success of inoculation of leguminous plants with rhizobia [16] resulting in the recognition of various factors affecting the survival and persistence of rhizobia in soil. These include antagonism and stimulation by other organisms, competition with other rhizobia, moisture, organic matter, pH, presence of host and other plants, predation, soil properties, and temperature (summarized by Beringer and Bale [17]). A major hamper in most early studies on microbial release was the lack of good markers to discriminate autochthonous and introduced strains. Since the introduced bacteria often survive at a low population density, close to, or even below the detection level, our insight in the ecology of rhizobia is still incomplete. The same holds for plant pathogens [18–20], human and animal pathogens [21–26], and microbes involved in the degradation of xenobiotics [27–29].

The Gus system as biomarker in rhizobia

Release of rhizobia is a common practice in agriculture to improve symbiotic nitrogen fixation in leguminous crops. When autochthonous strains are present in the soil, one has to discriminate the introduced strain from the local ones. Several methods have been used to identify specific strains in studies of rhizobial competition. In addition to the use of intrinsic antibiotic resistance, plasmid profile analysis and serology, we have examined the potential of the *Escherichia coli gusA* marker gene [30] in ecological studies. Due to the absence of background β -glucuronidase (GUS) activity in most plant tissue and microorganisms that interact with plants [31,32], GUS can be used as a very specific marker in plant–microbe interactions. The activity of the enzyme β -glucuronidase, which is encoded by the *gusA* gene, can, in the presence of a chromogenic GUS substrate, be clearly detected in a nodule infected by the marked strain. Rhizobia strains have been marked with the *gusA* gene, using the delivery plasmid pmTn5SS*gusA*10 (Sessitsch et al., in preparation). In our construct the *gusA* gene is under the *ptac* promoter and the *lacIq* gene. The *lacIq* gene product represses *ptac* and thus prevents expression of *gusA*. Derepression is achieved by the inducer molecule isopropyl- β -D-thiogalactoside (IPTG). The system makes use of the advantages of the miniTn5-Sm/Sp element [33] resulting in a stable and random genomic integration. This technology was used to compare the competitive abilities and symbiotic properties of *gusA*-marked rhizobia strains with its parent strains. Selection of recombinants that have similar properties as the parental strains can be used to study the ecology of the wild-type, parental strain (provided that release of the recombinant will be allowed).

Detection of uncultured microorganisms

Conventional isolation and cultivation methods are very sensitive techniques to detect microorganisms that easily grow in pure culture and that can be cultivated on selective media. Theoretically, each viable, living cell can be detected as a colony on a selective agar plate. By combin-

ing different cultivation techniques, it is possible to detect and identify very low numbers of microorganisms with these conventional methods. However, the method is unsuitable for recalcitrant or non-cultured organisms and organisms that cannot be selected on special media. During the last decade, alternative molecular techniques have been developed for rapid and direct detection and identification of microorganisms on the basis of specific nucleotide sequences in the DNA or RNA of these organisms. A common detection strategy may consist of the following steps: DNA/RNA extraction, in vitro amplification of a particular DNA/RNA fragment using the polymerase chain reaction (PCR), cloning of the PCR product, sequencing, comparison of the nucleic acid sequence with sequences in a databank, development of a specific oligonucleotide probe, and hybridization of the specific probe sequence to the nucleic acids extracted from an environmental sample. Some examples of the different steps in this strategy will be discussed in more detail.

Extraction of nucleic acids from environmental samples

During the last few years, an increasing number of technical papers have appeared which describe in detail how to obtain pure nucleic acids from environmental samples, including drinking water [34], sea water [35–39], fresh water [40,41], sediments [42,43], soil [13,35,42–50], actinorhizal root nodules [51], and many other types of environmental samples. The results of these methods are highly dependent on the type of environmental sample, the type of organism, and the additional steps required in the isolation procedure to obtain nucleic acids which are sufficiently pure to analyze. None of the described methods can be proposed as a universal analysis method.

The extraction of 16S rRNA from environmental samples may sometimes be preferred over the extraction of DNA, since 16S rRNA is often present in larger numbers. In addition, methods based on RNA detection may provide more relevant information with respect to the metabolic state of the isolated microorganisms. However, if

16S rRNA is being used as a target, different and often more elaborate extraction procedures must be used, since RNA is chemically less stable than DNA and is easily degraded by nucleases, which are often present in large amounts in environmental samples. Like in the DNA extraction procedures, most authors present their method as 'simple, rapid, accurate' and often also 'universal'. In practice, unfortunately, the opposite is frequently true, and each procedure has to be adapted to the type of environmental sample. For details see Stackebrandt and Goodfellow [52].

Nucleic acid probes

Application of nucleic acid probes in natural populations

The presence of target organisms can be detected by direct hybridization with a specific nucleic acid probe. Nucleic acid probes are nucleotide sequences complementary to the sequences in the target nucleic acid. The probes can either be DNA or RNA. Usually these probes are labelled with a radioactive reporter group (see [13,51]), but during the last few years there is an increasing interest for using non-radioactive probes (e.g. [25,53]). The probe signal obtained from the reporter group after hybridization and washing away the excess unbound probe, is proportional to the amount of target nucleic acid present in the sample.

DNA probes used in environmental studies may consist of relatively long cloned fragments of plasmids (e.g. [20,22,54,55]) or genes, such as cloned copper resistance genes [19,56], enterotoxin genes [57,58], and genes involved in the degradation of xenobiotics [29,59–62]. Alternatively, the probes may consist of short (usual 20–30) nucleic acid sequences defined as oligonucleotide probes.

Although these hybridization techniques are very useful, the lack of sensitivity is a major drawback, and microbial populations consisting of 10^6 per ml or lower usually cannot be detected accurately.

16S rRNA-targeted oligonucleotide probes to detect Frankia in actinorhizal nodules

16S rRNA-targeted oligonucleotide probes have been successfully used for detection of both cultured and uncultured microorganisms. Development of these probes is relatively easy, because the required sequence information can be readily obtained by PCR techniques. The PCR technique is particularly useful, since the 16S rRNA gene can be amplified by using primers against conserved regions in the 16S rRNA from diverse groups of bacteria. Detection of *Frankia* strains, the actinomycetous endophytes of nitrogen-fixing root nodules of non-leguminous (i.e. actinorhizal) plants, is greatly facilitated by the use of oligonucleotide probes. A genus-specific and several species/strain-specific probes have been developed, not only for pure cultures of strains [63–68] but also for those which have never been isolated from root nodules [68,94].

Use of polymerase chain reaction (PCR) to detect low numbers of bacteria

In order to be able to detect bacteria present in numbers below 10^6 per ml, the target DNA from these bacteria has to be amplified in vitro by the polymerase chain reaction. The PCR technique has proven to be very powerful and allowed to detect microbes down to a few cells per ml. In practice, however, again there are many drawbacks and the technique is not yet sufficiently standardized for quantitative use. In addition, PCR signals at low population density are difficult to interpret, because free DNA can also be amplified. The application is mostly qualitative and attempts are being made to quantify the PCR signals [38,42,51,58,69,70].

Survival of GEMs in the environment

Studying survival of GEMs in the environment requires sensitive and specific detection methods because low numbers of GEMs have to be detected in a background of biomass of autochthones [47,71–73]. Monitoring GEMs in the environment requires target molecules that are

specific for the GEM, and that are present in sufficiently large amounts to allow detection of GEMs which are present in low concentrations. Usually, the target molecule is (a part of) the recombinant DNA of the GEM itself, and can be amplified by the PCR and subsequently detected by hybridization or sequencing techniques. Alternatively, an additional reporter, or marker gene can be inserted into the DNA of the GEM. Some reporter genes can be detected on the basis of gene expression. Examples of the use of such reporter genes are antibiotic resistance genes, whose expression can be monitored on plates containing a selective medium, or with general hybridization techniques [74–76]. Other examples are catabolic genes involved in de chlorobenzoate degradation [77,78], recombinant plasmids [41, 42,79], and a deregulated monooxygenase [60]. Special attention is given to the detection of metabolic genes (marker genes), such as the *lacZY* gene, the *xylE* gene [72], the *lux* gene [80], and the *gusA* gene. In addition, a number of other specific genes have been used as marker genes, such as a cytotoxine-hemolysine gene [81], and herbicide resistance genes [28].

In order to increase the sensitivity of the method, unusual marker genes from eukaryotic origin have been inserted in the GEMs, e.g. the human growth hormone gene [82], cDNA for an egg yolk protein from *Drosophila* in *E. coli* [83], and the *pat* gene in *Pseudomonas* [84]. In order to obtain the highest sensitivity, a combination was made of using specific probes and PCR amplification techniques [21,42,58,69,70,85]. The reported detection levels in the referred papers again indicate that in practise the limits of detection of these new techniques should not be overestimated.

Effect of pollution on the diversity of soil microbial communities

All microorganisms capable of synthesizing antibiotics must have mechanisms to prevent suicide [86]. It is thought that many of the antibiotic resistance genes found in medically important bacteria originate from those organisms capable of producing the corresponding antibiotic [87].

Their movement is often facilitated by selection pressure exerted through the clinical and agricultural use of the particular antibiotic in question. It has been calculated that annually 30 000 kg of neomycin and kanamycin are ploughed into the top 5 cm of 75% of the agricultural land in the Netherlands [88], through the application of antibiotic 'fed' cattle manure to agricultural soils. The gene *aph*, encoding for aminoglycoside phosphotransferase, is one mechanism of resistance to neomycin and kanamycin. Seven classes of *aph* genes have so far been characterized, all of which share high levels of homology, indicating that one of those, the *aphV* gene, of the producing organism (*Streptomyces fradiae*), or other so far undiscovered actinomycetes provides the origin for other *aph* genes [89]. As almost all antibiotics are produced by bacteria of the genus *Streptomyces* [90], whose natural habitat is the soil environment, it is possible that there is a link between such agricultural practices and the appearance of similar antibiotic resistance genes in other bacterial genera. Recent work in Wageningen (Herron et al., in preparation) has shown that the addition of neomycin to soil exerts a powerful effect on the survival and gene transfer of resistant and sensitive strains of streptomycetes in situ. The use of genetically marked, well-defined strains will significantly simplify the detection of the microbes involved in this process.

Gene expression

All information, discussed above has been focussed on the detection of microorganisms on the basis of rRNA or DNA target sequences. This, however, gives no information on the expression of the genes, and does not always discriminate between nucleic acids from living or dead cells. Only recently, data became available on the detection of messenger RNAs (mRNAs) in environmental samples: human growth hormone [82], the *nptI* gene, the *rbcL* gene, encoding the CO₂-fixing enzyme, RuBisCo [91], and the mercury resistance gene [92]. Since the basic techniques for detection of mRNA in environmental samples are now available, it is tempting to suggest that it will become possible to detect gene expression in

microbial cells under environmental conditions (although half-life of mRNA is very short compared to eukaryotic rRNA).

Recent work in our research group has focussed on the localization of mRNAs from plant genes (Pawlowski et al., submitted) and bacterial genes [93,94] that are expressed in the early stages of infection of actinorhizal nodules. As an example of *Frankia* genes that are expressed in the root nodules of *Coriaria nepalensis* we chose *nifH*. Highly specific localization of the gene products was detected in palissade 'vesicles' micro-autoradiographically by using a ³⁵S-labelled probe that only hybridizes to the mRNA of the *nifH* gene.

Cloning and sequencing as new tools in molecular microbial ecology

Amplification of partial or complete genes by PCR is being used exclusively to detect and to identify bacteria in environmental samples. PCR can be made by using primers specific for a particular target organism in the sample. Alternatively, PCR products can be subjected to restriction fragment length polymorphism (RFLP) analysis, or probed with a specific oligonucleotide probe. However, use of PCR-amplified targets, especially from environmental samples, require the use of more than one probe or sequencing of various PCR products to avoid amplification of unrelated genes [94].

During the last 5 years an increasing number of papers has been published on 16S rRNA sequence data for phylogenetic research (see overview W. Ludwig and Schleifer [95]). Without ignoring the large contribution of many others, we will exemplify the approach with anaerobic propionate degradation which we study in our research group. Breakdown of propionate to methane and carbon dioxide is carried out by a syntrophic consortium of bacteria. The first propionate-degrading consortium was obtained by Boone and Bryant [96]. *Syntrophobacter wolinii* was described as a new obligately anaerobic Gram-negative rod which degrades propionate in co-culture with *Desulfovibrio* sp. The former could not grow in the absence of its partner, whilst the latter could. Phylogenetic characteriza-

tion of the bacterium became available by sequencing PCR amplification products of 16S rRNA genes of the members of the co-culture and focussing on the unknown sequences of *Syntrophobacter wolinii*. The results indicated that the bacterium was not related to bacteria growing synthrophically on other fatty acids than propionate, but was related to sulfate-reducing bacteria [97]. Probes have now been designed and are available for population ecological studies.

Concluding remarks

As has been shown before, population/community studies of microbes are often hampered by the techniques currently present to detect and identify the organisms in the environment. Unlike higher plants and animals, bacteria seldom have a characteristic morphology and can better be detected and identified on a molecular level. Many of them refuse to grow under the artificial conditions we have chosen in our experiments. The introduction of new molecular techniques has enabled us to re-investigate neglected 'uncultured' organisms on the basis of phylogenetic data. A direct identification of such organisms at the nucleic acid level definitely helps us to improve our knowledge on the diversity of the natural microflora.

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