Review TRENDS in Biotechnology Vol.24 No.7 July 2006

Homogeneous versus heterogeneous probes for microbial ecological microarrays

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Microbial ecological microarrays have been developed for investigating the composition and functions of microorganism communities in environmental niches. These arrays include microbial identification microarrays, which use oligonucleotides, gene fragments or microbial genomes as probes. In this article, the advantages and disadvantages of each type of probe are reviewed. Oligonucleotide probes are currently useful for probing uncultivated bacteria that are not amenable to gene fragment probing, whereas the functional gene fragments amplified randomly from microbial genomes require phylogenetic and hierarchical categorization before use as microbial identification probes, despite their high resolution for both specificity and sensitivity. Until more bacteria are sequenced and gene fragment probes are thoroughly validated, heterogeneous bacterial genome probes will provide a simple, sensitive and quantitative tool for exploring the ecosystem structure.

Introduction

Microorganisms are everywhere on earth. Understanding the structure and composition of microbial communities is crucial to maintaining a desirable ecosystem function and beneficial to human health. For two decades the paradigm of the 'great plate count anomaly' [1] has guided a change in the tools used by microbial ecologists away from agar media towards PCR-based approaches [2–4], including sequencing of the rRNA genes, denaturing gradient-gel electrophoresis (DGGE), terminal restriction-fragment length polymorphism (tRFLP), quantitative real-time PCR and fluorescence *in situ* hybridization (FISH) [3,5–8]. Using these techniques, many microbial structures in various ecosystems, including the gastrointestinal tract of higher organisms, have been partly revealed. However, these PCR-based molecular procedures are labour-intensive, time-consuming and can introduce certain biases, such as Taq errors [9] and skewed template-to-product ratios [10]. Thus, additional high-throughput and hybridization-based quantitative methods for analyzing the bacterial involvement in ecosystems are warranted. DNA microarrays, originally developed for exploring genome-wide transcriptional profiles, are now broadly applied across most sectors of the life sciences, including environmental microbiology and microbial ecology [11,12]. The microarrays developed for investigating the composition and function of microorganisms in an environmental niche are known as environmental microarrays, microbial diagnostic microarrays or microbial ecological microarrays. Of these, microbial ecological microarrays are classified as either microbial identification microarrays or functional gene microarrays, according to their objectives [11]. Furthermore, microbial identification microarrays, developed for investigating the microbial structures of ecosystems, can be further divided according to their probe types: oligonucleotides, gene fragments and complete or partial microbial genomes (Table 1). Given that genome probes are composed of various genes, contrary to oligonucleotides and gene fragments, which are fabricated as a single molecule, they could be defined as heterogeneous microarray probes, and oligonucleotides and gene fragments could be defined as homogeneous probes. In this review, the advantages and disadvantages of each type of microbial ecological microarray probe are discussed with regard to appropriate applications of microarrays in microbial diagnostics and ecology.

Gene fragments as microbial ecological microarray probes

Tiedje and co-workers [13] pioneered a method based on using unsequenced, random genome fragments as DNA microarray probes for discriminating among *Pseudomonas* species; this method is recognized as a classic approach for microbial ecological microarrays. Through using probes comprising randomly generated gene fragments, Kim *et al.* recently presented the successful diagnosis of different bacteria in activated sludge [14]. Gene fragment probes are of great interest because most current microbial ecological microarrays use 20–70mers of 16S rRNA as gene-targeting probes – these have low resolution for species differentiation [15–17] and low sensitivity without PCR amplification of the targeted environmental bulk DNA [11]. Kim *et al.* avoided the shortcomings of oligonucleotide microarrays by using gene fragments in the range of 200–1500 bp as probes [12], and

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Available online 12 May 2006

 0167-7799/$ - see front matter doi:10.1016/j.tibtech.2006.04.010

Table 1. Major differences in microbial ecological microarray probes

Probe type	Probe size	Sensitivity*	Fabrication method	Advantage	Disadvantage
Oligonucleotide	20–70bp	25ng	*In silico* design and oligomer synthesizer	Probing unculti-vated communities Easy probe design	Low sensitivity Low specificity
Gene fragments	200–1000bp	1ng	*In silico* primer design and PCR	Hierarchical probes High sensitivity	Genome-seqeuncing Formidable validation
Bacterial genome	Entire genomic DNAs (3–7 Mbp)	0.25ng	Cultivation and genome extraction	High sensitivity High specificity	Low-throughput obtain-ing probes and only probe isolated bacteria

*Sensitivity: the amount of the labelled, pure, genomic DNA that can be detected.

overcame the low resolution by targeting functional genes rather than the most evolutionarily conserved 16S rRNA genes [18]. The possibility that a 16S rRNA gene is amplified in a random amplification of a bacterial genome is $<1\%$, given that between 1 and 7 operons of 16S rRNA are present in a bacterial genome ranging between 3–7 Mb. Currently, the specificity of a microbial diagnostic microarray is evaluated and categorized using a phylogenetic, hierarchical taxonomic system such as genus-, species-, subspecies- or strain-specificity [17,19]. Despite randomly generated genomic DNA probes originally being developed for detecting the presence of specific bacterial strains, it is difficult to evaluate them with regards to species- or strain-specificity. Additionally, although the use of an appropriate number of unsequenced probes might eliminate the time and expense of sequencing [14], the uncertainty in microarray probes could cause several problems. Bacterial genomes contain various genes evolving at different rates [20]. Thus, in a microarray experiment, highly conserved genes, such as 16S rRNA genes, might hybridize to genes of a different genus [17] and some functional genes might not hybridize to any gene in closely related strains of the same species [21]. The average amino acid identity between two species sharing in the range of 95–97% 16S rRNA gene identity is between 56% and 76% [22] (and much lower if calculated by nucleotide identity). Given that non-target genes with more than 87% identity to probes hybridize under general microarray hybridization conditions [11], most of the randomly generated genomic DNA probes might be species-specific and some would be strain-specific; therefore, the specificity of every randomly generated probe must be clearly categorized hierarchically. Furthermore, all randomly generated probes must be sequenced and more precisely evaluated to be reproducible by other researchers.

Significant differences in gene content and genome size occur among multiple strains from the same species, and gene fragment probes can be used to detect these differences without the need for sequencing additional genomes [23]. This approach has been used successfully to detect genomic intraspecies diversity among multiple strains of *Campylobacter jejuni*, *Helicobacter pylori*, *Salmonella* sp., *Vibrio cholerae*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and *Escherichia coli*. Strain-specific genes often include putative virulence factors, factors involved in host interaction and mobile genetic elements, whereas conserved genes encode most of the metabolic and cellular processes. With the advantage of high sensitivity, hierarchically categorized gene fragment probes can be used to reliably detect and/or identify, at the species, subspecies or strain levels, one or a few microbes out of the many that might be present.

Oligonucleotides as microbial ecological microarray probes

Analysis of the rRNA gene sequence originally developed to provide a universal phylogeny of life forms has proven useful in many areas of biological research. As a step toward overcoming the limits to the rate at which sequences can be analyzed, Guschin *et al.* first described and demonstrated the concept of oligonucleotide chips for microbial detection using polyacrylamide gel micropads with nine immobilized oligonucleotides to discriminate various ammonia-oxidizing bacteria [24]. Recently a high-density Affymetrix GeneChip™, containing more than 30 000 16S rRNA-targeting oligonucleotide probes, has been developed for identifying bacterial species and subsequently characterizing populations of airborne bacteria at the level of higher phylogenetic taxa [25]. The oligonucleotide probes have several advantages over the other formats: a large number of uncultivated microorganisms can be probed, oligo-probes can be designed for high-throughput using probe-designing software, and fabrication can be ordered at low cost from oligo-synthesizing manufacturers. However, oligonucleotide probes provide poor resolution at the species level in some bacterial groups [15,16,26]: multiple rRNA operons in various genomes (ranging from 2–13), and the intragenomic sequence heterogeneity of 16S rRNA genes (ranging from 95–100%), make it difficult to fabricate high-resolution probes. Owing to the low sensitivity of oligonucleotide probes, most microarray researchers, until recently, have used PCR amplification for detecting genes from the natural environment. However, PCR-based microarrays have been criticized because PCR amplification of environmental DNA produces biased template-to-product ratios and, as a result, fails to reflect the community composition quantitatively [27]. The heterogeneity of 16S rRNA among the multiple copies in a strain can also sometimes mask the appropriate probe for a specific species.

The low sensitivity of oligonucleotide probes might become less crucial as dye chemistry [28], scanning technology [29] and microarray image processing [30] are further developed. Rather, the specificity of oligonucleotide probes is the crucial issue – the fabrication of a 16S rRNA-targeting oligonucleotide probe that is precisely specific for a certain species is a formidable task. The homology of 16S rRNA gene sequences among bacterial species is variable, even among close relatives (Box 1). Thus, it is impossible to apply standard criteria for species-specific probe design software such as ARB [31] or HPD [19]; rather, with these software, we must designate a group of strains (nodes in a phylogenetic tree) to fix a species. Despite these efforts, our team frequently failed to obtain a species-specific probe because of the highly conserved character of 16S rRNA genes. Given that some gene fragment probes can be strain-specific, this could be achieved with an oligonucleotide probe that did not target 16S rRNA genes but targeted several functional genes. The fundamental reason for the failure of species-specific 16S rRNA gene-targeting oligonucleotides is that fixed similarities in 16S rRNA genes do not demarcate a bacterial species, and novel species are not identified by the results obtained with an oligonucleotide probe (Box 1). Species-specific primers, which are widely used in quantitative real-time PCR, present the same problem when used for multiplex diagnostics. Although alternative, higher-resolution universal marker genes, including *rpoB*, *recA*, *gyrB*, *groEL*, *atpD* or the *tmRNA* gene [4], are now used as oligonucleotide probes, these have similar disadvantages to species-specific probes.

Given that species-specific probes are essentially group-specific probes, many species-targeting probes frequently miss or falsely identify strains belonging to a bacterial species. Because bacterial species have been demarcated by probing with existing bacterial genomes rather than oligonucleotides, the species specificity of oligonucleotide probes needs to be rigorously validated with many strains. Although discrimination between a perfect match and a single mismatch is considered the ultimate specificity in bacterial diagnostics, such probes also need to be validated with all known strains of a species and its closest relatives. Because the 16s rRNA gene clones from uncultivated bacteria are not validated as belonging to a species in current bacterial systematics, the probing capability of oligonucleotides from these clones is not species specific, despite its great usefulness. When 16S rDNA-based oligo-probes are designed with ARB, frequent updating with sequences from databanks such as the NCBI would be helpful for validating the specificity of a designed probe.

Box 1. Current concept of bacterial species

By contrast to higher organisms, which can be classified into taxonomic groups based on the ability to interbreed, there is no fixed criterion for bacterial species demarcation other than definitions based on human interests such as disease-associated bacteria. Currently, a prokaryotic species is defined as 'a genomic coherent group of strains sharing a high degree of similarity in independent features' [40]. Practically, a prokaryotic species is considered as group of strains with DNA–DNA hybridization (DDH) ratios >70%, and >97% 16S rRNA gene-sequence identity [41]. Since the early 1970s, prokaryotic species delineations are based entirely on data generated by DDH experiments because bacteria with more than 70% genomic DDH ratios have not been observed to have less than 97% 16S rRNA gene-sequence identity. In this approach, the overall genetic similarity among isolates is assessed by the degree to which their genomes hybridize under standardized conditions. Interestingly, many biologists found that most bacterial strains had DDH values that were above 70% or below 30%, which might be explained by population genetics such as the 'rugged fitness landscape model' [42]. Although DDH experiments remain the cornerstone of present-day bacterial taxonomy, they have several shortcomings. Besides problems associated with the performance and reproducibility of DDH experiments, experimentally determined genomic similarities do not represent actual sequence identities because DNA heteroduplexes form when the two strands have at least 80% sequence identity [42]. Therefore, a difference of 20% in sequence identity might lead to DNA reassociation of between 0% and 100% [43,44]. Recently, however, Konstantinidis and Tiedje [45] compared the average nucleotide identity (ANI) of the shared genes in two strains with their DDH ratios. In this report, ANI shows a strong linear correlation to DNA–DNA reassociation values. Furthermore, Henz *et al.* [46] recently used a novel strategy called 'genome blast distance phylogeny (GBDP)' to derive phylogenies based on the entire genomic information of organisms. These genomics-based approaches will dispel the vagueness of DDH and make DDH experiments a more convincing method for bacterial species demarcation.

Microbial genomes as microbial ecological microarray probes

Most microbial ecological microarrays use oligonucleotides as probes, and these have been extensively investigated and continually improved [18,19,26]. However, recently, microarrays using microbial genomes as probes have been developed, which have led to advances in the specific, sensitive and quantitative monitoring of microbial dynamics in natural environments [21,32,33]. Zhang *et al.* reported the 'library on a slide', a microarray fabricated with an *E. coli* reference collection, for exploring genetic diversity within the species [34], and this 'array of diverse microbial genomes' has been examined for its potential to study complex microbial systems [35]. This study was based on a previously developed membrane-based macroarray for reverse-sample genome probing (RSGP) [36], which has been applied extensively to exploring the microbial communities in oil fields. The first application of microarrayed bacterial genomes on a glass slide for microbial ecology was accomplished by Wu *et al.* [33]. In their work, the specificity, sensitivity and quantitative aspects of genome probes were evaluated, intensively, within the context of environmental applications, in addition to their potential for bacterial classification. However, there remains a need to explain the background rationales for the phylogenetically variable specificity of genome probes, depending on hybridization temperatures. Using greatly advanced sensitivity, Bae *et al.* applied the genome probes to monitoring the dynamics of ~150 lactic acid bacteria in food fermentation [32]. However, the phylogenomic analysis and nucleic-acid hybridization chemistry of the species specificity of genome probes needs further investigation.

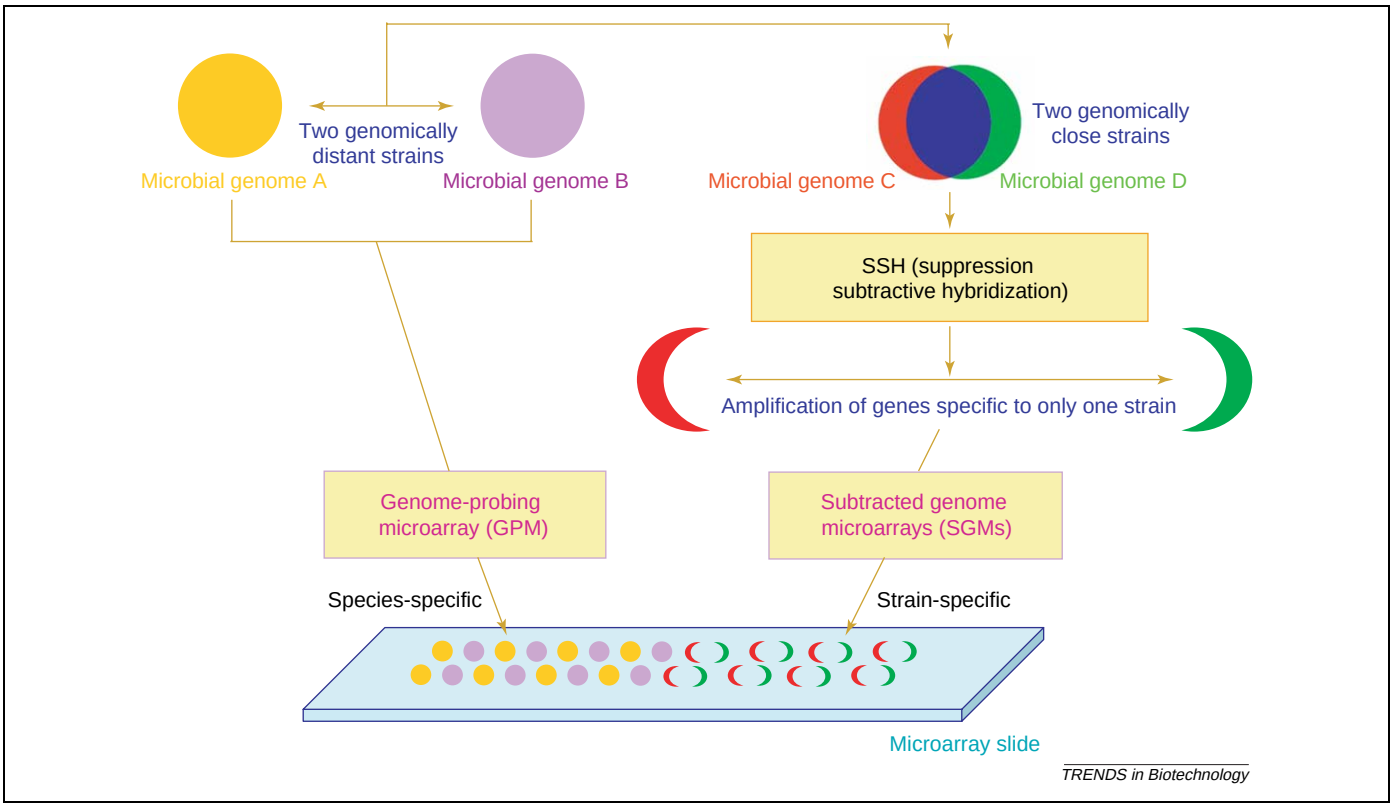

Figure 1. A schematic diagram of two representative applications of genome probes. Although genome-probing microarrays use entire bacterial genomes as microbial ecological microarray probes for species-specific diagnosis, subtracted genome microarrays use partial genomes, following subtraction of the genome of the phylogenetically closest bacteria, for strain-specific diagnosis.

The different cross-hybridization ratios obtained from membrane-based approaches also needs to be validated rigorously. The problem with 16S rRNA genes, the usual suspects in cross-hybridization, could be solved if bacterial transcripts could be harvested, reverse-transcribed, dye-labelled and hybridized to genome microarrays. This would be a crucial step toward the application of genome probes as an 'environmental transcriptomics' tool [37].

Using a previously developed method, subtractive suppression hybridization (SSH), Bae *et al.* subtracted the bacterial genome probes that could hybridize with phylogenetically close relatives to achieve better strain-specificity with genome probes (Figure 1) [21]. In a similar way to transcriptome hybridization, this approach could help eliminate the species specificity defect of genome probes. However, it is an open question as to whether a mixed SSH pool is the best way to differentiate strains. Although it clearly worked in the reported examples, one or several homogeneous probes were ultimately required to diagnose a bacterial strain because the subtracted genome contains uncertainties and is difficult to reproduce. Given that the bacterial genome subtracted with a phylogenetically close relative did not show cross-hybridization, the SSH-generated genome must be composed of strain-specific genes. Therefore, as with gene fragment probes, all of these genes should be sequenced and validated for the investigation of intraspecies diversity and entities that cause disease in the strain.

Future directions for microbial ecological microarray probes

Increasing numbers of microbial genomes are being sequenced and fabricated as microarrays for exploring genome-wide transcriptional profiles. Most of these single genome microarrays are manufactured with the gene fragment probes. Although these probes are useful for investigating genomic intraspecies diversity among multiple strains of a species with high sensitivity, phylogenetic and hierarchical categorization of all the gene fragment probes is a too formidable task. Furthermore, the speed of microbial genome sequencing so far is not enough to use the gene fragments arrays in environmental or ecological studies. Despite their low sensitivity, oligonucleotide probes are currently the most useful way for diagnosing the uncultivated bacteria that occupy the major part in environment. Future technical and bioinformatic developments will inevitably improve the potential of oligonucleotide probes further [26]. Until many more bacterial genomes have been sequenced and gene fragments probes thoroughly validated, heterogeneous bacterial genome probes will provide a simple, sensitive and quantitative tool for exploring the ecosystem structure. The fundamental problem that genome probes cannot be used to probe uncultivated bacteria can be solved if the genomic DNA is amplified from single, flow-sorted bacterial cells by the multiple displacement amplification (MDA) reaction using phi 29 DNA polymerase [38]. Although the metagenome is always the

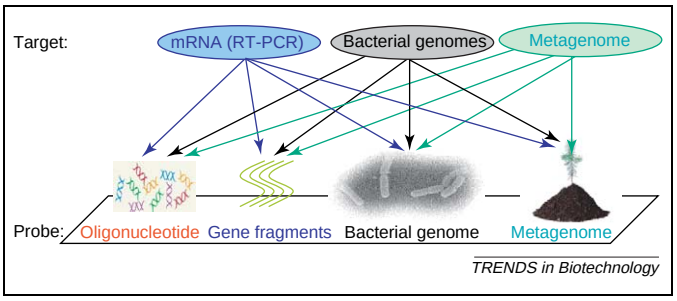

Figure 2. Current and future strategies for microbial ecological microarray probes. Most microarrays developed for gene-expression profiling are spotted with oligonucleotides or gene fragments and hybridized with a bacterial transcriptome or genome. Bacterial genome–genome hybridization is mainly used for bacterial species demarcation (Box 1). To date, most microbial ecological microarray hybridizations are between the target metagenome and homogeneous probes such as oligonucleotides or gene fragments. Recently, bacterial genomes have been used as a type of heterogeneous microarray probe to increase species specificity and sensitivity. Bacterial transcriptome hybridization with microbial ecological microarray probes should prove to be a crucial step toward monitoring real bacterial dynamics. Metagenomes also could be used as heterogeneous microarray probes.

target in microbial ecological microarray hybridizations, it can be used as a heterogeneous microarray probe, similar to the 'array of diverse microbial genomes' [34] when biotechnologically useful materials, such as polyketide synthases or peptide synthetases, are pursued with sequence-driven analysis [39]. Metagenome–metagenome hybridization might be applied to comparisons of microbial communities, such as fluctuations in the normal gastrointestinal flora caused by some foods (Figure 2).

Acknowledgements

The authors are supported by grant BDM0200524, NNM0100512, NRL research program (NLW0070524) and KRIBB Research Initiative Program from the Ministry of Science and Technology (MOST) of the Republic of Korea.

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