A2.

Appendix 2

Environmental genomics: exploring the unmined richness of bacteria to degrade xenobiotics

Environmental genomics: exploring the unmined richness of bacteria to degrade xenobiotics

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A2.1. Abstract

Increasing pollution of water and soils by xenobiotic compounds has led in the last few decades to an acute need for understanding the impact of toxic compounds on microbial populations, elucidation of catabolic degradation pathways of xenobiotics as well as set-up and improvement of bioremediation processes. Recent advances in molecular techniques including high-throughput approaches such as microarrays and metagenomics have opened up new perspectives and pointed towards new opportunities in pollution abatement and environmental management. Compared to traditional molecular techniques dependent on the isolation of pure cultures in the laboratory, microarrays and metagenomics allow answering specific environmental questions by exploring and using the phenomenal resources of uncultivable and uncharacterized microorganisms. This paper reviews the current potential of microarrays and metagenomics to investigate the genetic diversity of environmentally relevant microorganisms and identify new functional genes involved in the catabolism of xenobiotics.

A2.2. Introduction

Xenobiotics are defined as compounds that are foreign to a living organism. These molecules are not easily recognised by existing degradative enzymes and tend to accumulate in soils and water. Among other xenobiotics, polyaromatic, chlorinated and nitroaromatic compounds were shown to be toxic, mutagenic and carcinogenic for living organisms. Nevertheless, thanks to their diversity, versatility and plasticity for adaptation, microorganisms are the best candidates among all living organisms to funnel xenobiotic compounds into natural biogeochemical cycles. Indeed, more and more microorganisms are being described as able to degrade these anthropogenic molecules. However, some xenobiotics have been shown to be unusually recalcitrant, i.e. microorganisms either do not metabolize these xenobiotics or transform them into metabolites that accumulate. For example, in the case of nitroaromatic compounds and 2,4,6-trinitrotoluene (TNT) in particular, several species (mainly *Pseudomonads* and *Clostridia* species) have been isolated as able to transform TNT through characterized metabolic pathways into products, which are however as toxic as the parent molecule (Esteve-Nunez et al. 2001). Therefore, the discovery of new catabolic pathways leading to complete mineralization of the pollutant would be more valuable. In addition, the degradation pathways of many other xenobiotics remain to a large extent poorly characterized, if not totally unknown. A better knowledge of the diversity of catabolic pathways for the degradation of xenobiotics would certainly bring valuable information for bioremediation processes.

One of the reasons why our view of microbial degradation pathways is so incomplete lies in the continual isolation of the same species when cultivation of microbes degrading pollutants is attempted. The large majority of the earth's microorganisms remains indeed uncharacterized because of our inability to isolate and cultivate them in/on appropriate media. Although cultivation techniques are improving and have allowed to grow *in vitro* an increasing number of still uncultured microorganisms (Leadbetter 2003), our knowledge of their growth conditions in nature (i.e. chemistry of the original environment, life in complex communities, obligate interactions with other organisms, etc.) remains insufficient to cultivate most of them. This is particularly true in complex biological systems like soils, where, despite a huge bacterial diversity (up to 10^{10} bacteria and probably thousands of different species per

gram of soil (Rosselló-Mora and Amann 2001)), less than 1% of bacteria have been cultured so far (Torsvik and Øvreås 2002).

For the last 20 years, different molecular methods independent of cultivation have been developed to explore the diversity of microorganisms, cultivable or not, in natural environments. Most of these methods are based on PCR amplification and subsequent analysis of bacterial rRNA genes by fingerprint methods (clone libraries, RFLP, DGGE, etc.) and sequencing. The discovery of many new bacterial lineages as well as the reassignment of the most ecologically significant groups when using these methods have led to a dramatic change in our perception of microbial diversity and the phylogenetic tree of life (Ward et al. 1990; Amann et al. 1995).

However, even though our understanding of bacterial diversity is increasing thanks to the study of 16S rRNA genes, little is known about other protein-coding genes. Accessing the genes of uncharacterized microorganisms presents a tremendous potential for the discovery of new antibiotics, secondary metabolites or xenobiotic degradation pathways. This is illustrated by a recent work of Venter (2003): by using a cultivation-independent molecular approach, he found thousands of new bacterial species and more than one million new protein-coding genes in 200 liters of Sargasso seawater.

Powerful molecular methods have been recently developed to explore simultaneously the astonishing taxonomic *and* functional variety of environmental microorganisms like metagenomic libraries and microarrays. In the first methodology, libraries from the environmental metagenome are constructed and clones are screened for a desired trait (‘function-driven’ approach) or for a specific sequence (‘sequence-driven’ approach) (Schloss and Handelsman 2003). In the second methodology, thousands of designed DNA/RNA probes are spotted on a chip that can then be used to monitor the response of microorganisms to environmental changes. Alternatively, spotted probes may consist in clone libraries of environmental strains of interest.

Needless to say, environmental biotechnology is entering a new era with these recent molecular techniques. Several aspects of environmental biotechnology may benefit from these techniques, spanning the spectrum from environmental monitoring (Guschin et al. 1997) to bioremediation and biodegradation (Dennis et al. 2003). The objective of this mini-review is

to highlight the potentialities of new molecular approaches in exploring the genetic diversity of microbes to degrade xenobiotics.

A2.3. Metagenomic libraries

Metagenomics is the culture-independent genomic analysis of entire microbial communities (Schloss and Handelsman 2003). In other words, metagenomics provides access to the pool of genomes of a given environment. Furthermore, direct genomic cloning offers the possibility to retrieve unknown sequences or functions, whereas methods relying on PCR amplification require prior knowledge of the sequence of genes for the design of primers.

To build metagenomic libraries, total genomic DNA is extracted from the environment (Figure 1.a). Then, genomic DNA is enzymatically or mechanically fragmented. Fragments can be separated on the basis of their size and purified by pulsed field gel electrophoresis. In this way, fragments of appropriate size can be isolated from the gel and inserted in host cells by cloning vectors (bacteriophage lambda, cosmid, fosmid or BAC vectors). BAC (Bacterial Artificial Chromosome) vectors are especially efficient in maintaining stably large DNA inserts in low copy numbers in the host cells (1 to 2 per cell) (Shizuya and Simon 1992; Rondon et al. 2000).

Metagenomic libraries can be screened for functional and/or genetic diversity. The 'functional' approach is more appropriate for finding new catabolic genes for the degradation of xenobiotics. It consists in screening clones that express a desired trait on appropriate media. For example, chloroaromatic compounds could be used as sole electron acceptors since it has been shown that bacteria can respire them (El Fantroussi et al. 1998; van de Pas et al. 2001), whereas polyaromatic hydrocarbons could be utilized as sole C- and energy-source. Consequently, growth measurements could identify clones bearing catabolic genes.

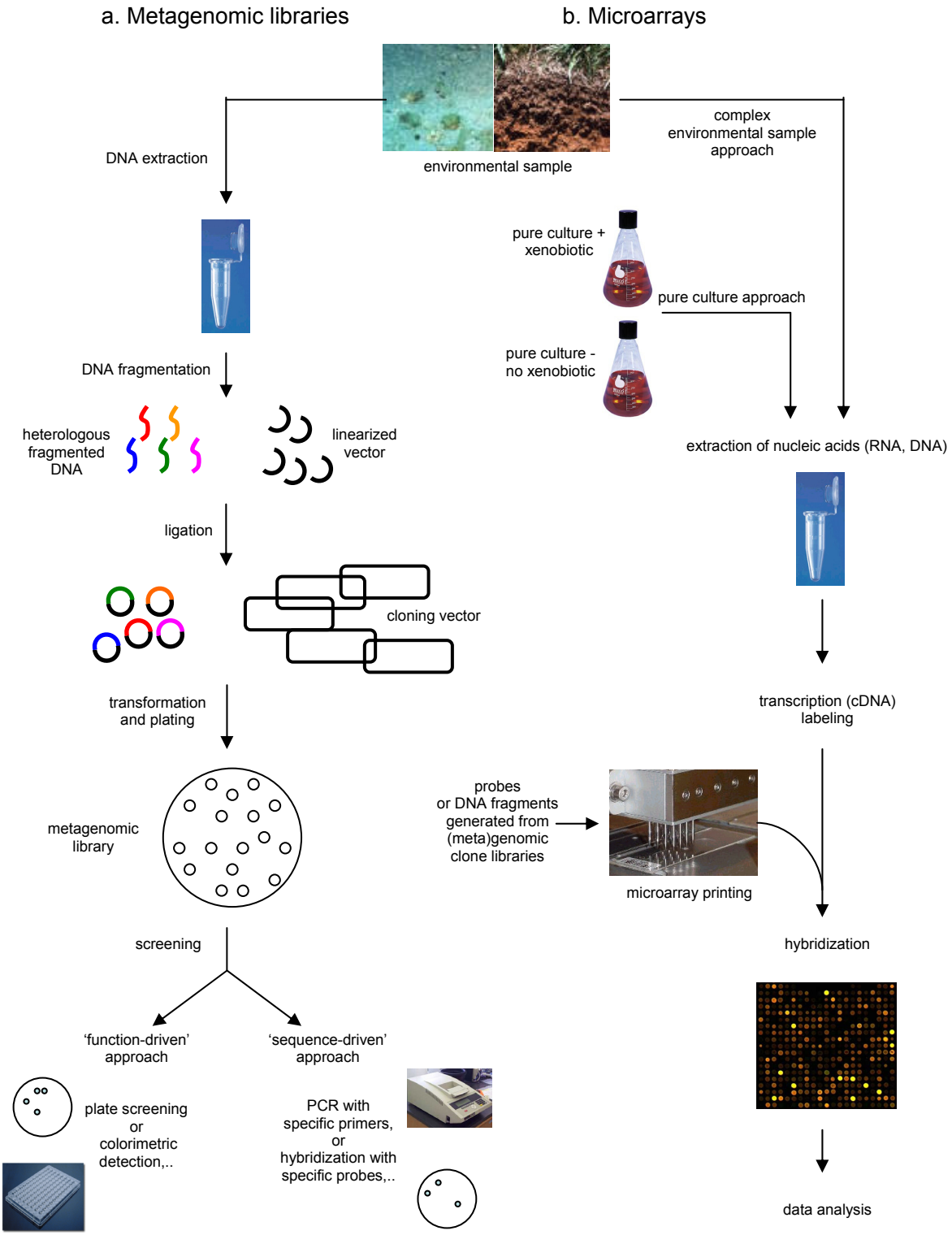

Figure 1.a. Construction and analysis of metagenomic libraries from environmental samples. b. Construction of microarrays and hybridization with complex environmental samples or pure cultures.

The main limitation of this method can be the low recovery of active clones, because the function is not always expressed in the host cell, especially when all the genes required for the function are not clustered. However, this function-driven screening remains a straightforward and successful method for the discovery of catabolic genes, as opposed to inferring the function of cloned genes by searching for homologous sequences available in databases (Rondon et al. 2000). Indeed, sequences coding for important metabolic functions are frequently poorly conserved, making the comparison of clone sequences with homologous ones very difficult. This functional screening approach has been successfully used to retrieve novel and previously undescribed genes coding for antibiotics, lipases, enzymes for the metabolism of 4-hydroxybutyrate, and genes encoding biotin synthetic pathways (Schloss and Handelsman 2003). One major advantage of using this cloning method lies in the fact that biodegradation genes are usually clustered in small genes, in contrast to biosynthetic pathways that require big clusters over 100 Mb. For example, genes involved in the biodegradation or biotransformation of nitroaromatic compounds are clustered in 5 to 27 kb fragments (Table 1). This table also illustrates the fact that most of the catabolic genes known to date were isolated from culturable microorganisms, after construction of genomic libraries and screening for a function (i.e. transformation of the toxic compound or production of metabolites) or for a sequence (i.e. with probes designed from already known sequences of catabolic genes). Up to now, no genes for TNT denitration have been discovered whereas recent data of our group have shown that high denitration activities can be obtained under defined conditions with a TNT-contaminated soil sample (Eyers et al. 2004). Moreover, the presence of a specific bacterial consortium in this polluted soil was demonstrated by Denaturing Gradient Gel Electrophoresis (Eyers et al. 2004). Therefore, metagenomic libraries are particularly promising for finding denitration genes compared to methods based on the isolation of pure cultures.

By contrast to the function-driven screening of metagenomic libraries, the 'sequence-driven' approach is based on conserved regions in microbial genes. Clone libraries are screened for specific DNA sequences by means of hybridisation probes and PCR primers, whose design is based on the information available in databases. Such hybridisation probes may consist, in the case of denitration of 2,4,6-trinitrophenol or other electron-deficient aromatics, in *ndpG* and *ndpI* genes of *Rhodococcus erythropolis* HL-PM1 (Table 1) as homologous genes were identified in other *Rhodococcus*-degrading strains (Heiss et al. 2003). These genes are clustered separately from related enzymes. Hence, it was suggested that they may be suitable

as gene probes for finding bacteria in the environment with the capacity to 'hydrogenate' electron deficient aromatic ring systems (Heiss et al. 2003).

Table 1. Properties of identified genes involved in degradative pathways of recalcitrant nitroaromatic compounds

Target compound	Name	Target metabolite in the pathway	Function	Size	Microorganism of origin	Mode of isolation	References
4-nitrotoluene	*ntnMA* *ntnD* *ntnC*	4-nitrotoluene 4-nitrobenzyl alcohol 4-nitrobenzaldehyde	Monooxygenase nitrobenzyl alcohol dehydrogenase nitrobenzaldehyde dehydrogenase	genes clustered in a 14.8 kb fragment	*Pseudomonas* sp. TW3	genomic library screened with designed probes; activities confirmed by cloning and expression in *E. coli*	(James and Williams 1998; James et al. 2000)
	PnbA *PnbB*	4-nitrobenzoate 4-hydroxylaminobenzoate	nitrobenzoate reductase hydroxylaminobenzoate lyase	genes clustered in a 6 kb fragment	*Pseudomonas* sp. TW3	genomic library expressed in *P. putida* PaW340 and screening for metabolic activities	(Hughes and Williams 2001)
2-nitrotoluene	*ntdAa* *ntdAb* *ntdAc* *ntdAd*	2-nitrotoluene	Reductase Ferrodoxin iron sulfur protein α iron sulfur protein β	genes clustered in a 4.9 kb fragment	*Pseudomonas* sp. JS42	genomic library expressed in *E. coli* and screening for metabolic activities	(Parales et al. 1996)
2,4-dinitrotoluene	*dntA* *dntB* *dntD* *dntG* *dntE*	2,4-dinitrotoluene 4-methyl-5-nitrocatechol 2,4,5-trihydroxytoluene 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadecanoic acid Methylmalonic acid semialdehyde	Dioxygenase Monooxygenase Dioxygenase isomerase/hydrolase Dehydrogenase	genes clustered in a 27 kb fragment	*Burkholderia cepacia* R34	genomic library expressed in *E. coli* and screening for metabolic activities	(Johnson et al. 2002)
2,4,6-trinitrophenol	*npdI* *npdG* *npdC*	2,4,6-trinitrophenol hydride-Meisenheimer complex of 2,4,6-trinitrophenol 2,4,6-trinitrophenol and its hydride-Meisenheimer	hydride transferase hydride transferase NADPH reductase	genes clustered in a 12.5 kb fragment	*Rhodococcus erythropolis* HL PM-1	genomic library and selection of clones thanks to mRNA differential display experiments	(Walters et al. 2001; Heiss et al. 2002)
2,4,6-trinitrotoluene	*xenB*	2,4,6-trinitrotoluene	Reductase	1.05 kb	*Pseudomonas fluorescens* I-C	genomic library expressed in *E. coli* and screening for metabolic activities	(Blehert et al. 1999)

A2.4. Challenges of metagenomic libraries

Although metagenomic libraries constitute at present the most powerful tool to assess the functional diversity of natural microbial communities, they do not cover genomes of low abundance in complex environments like soils, which can be, nonetheless, responsible for crucial degradation or related processes. As a result, the frequency of clones of a desired nature in a library can be very low. This implies screening of thousands of clones that can be laborious and time-consuming. High-throughput equipment are now available for facilitating colony picking, inoculation in microtiter plates and screening of numerous clones. Nonetheless, an enrichment strategy can be applied before the construction of the library to select for a specific feature and in that way better cover a subset of the community (Entcheva et al. 2001). This aspect is of particular interest for the genes of biodegradation pathways. Communities enriched naturally by long term exposure to high concentrations of xenobiotics may host the genes of interest at high frequency. Currently we are screening a fosmid library constructed from soils heavily polluted with TNT (20-30 g/kg) for more than 20 years. Pools of genes of interest might also be enriched by the classical enrichment techniques in the laboratory. In this context metagenomics might shorten the time required to understand the genetics of degradation.

Moreover, the catabolism of specific xenobiotics may be achieved by two or more bacteria, each species contributing to part of the catabolic pathway, as it is the case for polychlorinated biphenyl compounds (Abraham et al. 2002). In this case, it will not be possible to isolate on a contiguous piece of DNA all the genes involved in the catabolic pathway. Therefore, Schloss and Handelsman (2003) suggested studying multiple clones simultaneously (instead of individual clones) on liquid media in which substrates and products can diffuse freely among members of the mixture.

Finally, another challenge lies in choosing an appropriate host of expression. Indeed, it is crucial for functional screening that catabolic genes are effectively expressed. In addition, the host has to be relatively insensitive to the toxic xenobiotic as well as unable to catabolise it in the absence of the vector.

A2.5. Microarrays

The genetic information, accumulated by major research programs such as the sequencing of the human genome and genomes of hundreds of other organisms, opens access today for the scientific community to the systematic study of the organization of regulatory networks at the cellular level. An emerging technique making it possible to analyse hundreds and even thousands of genes at the same time lies in the DNA chip, thus getting away from the "one gene at a time" analysis. For these studies, extremely small amounts of biomolecules, (RNA, cDNA,…) are printed at high density on substrates.

Compared to traditional nucleic acid membrane hybridization, microarrays present the advantage of miniaturization (thousands of probes can be spotted on a chip), high sensitivity and rapid detection. As pointed out by Zhou and Thompson (2002), microarray-based genomic technologies are bound to revolutionize the analysis of microbial community structure, function and dynamics.

DNA microarrays are coated glass microscope slides onto which thousands of target DNA samples have been spotted in a precise pattern. There are two principal microarray types based on the nature of the target: DNA (cDNA, PCR products, oligonucleotides or plasmids) or RNA, and on the method of spotting DNA: mechanical microspotting or photolithography. In the first method, purified DNA is spotted onto membranes or coated glass slides. Though DNA will stick to the glass, aminosilane-coated and poly-L-lysine (PLL) coated slides are predominantly used. Silanized slides ($RSiX_3$, X typically alkoxy) attach DNA by forming covalent bonds between primary amines on the surface and the phosphate backbone (Celis et al. 2000; Ye et al. 2001). The photolithography method uses an ultraviolet light source that passes through a mask where a photochemical reaction takes place on a siliconized glass surface (Affymetrix). This is by far the most efficient method of generating high-density oligonucleotide chips, but has practical limitations in terms of fragment length and affordability (Kumar et al. 2000).

After printing of microarrays, the next step consists in extracting DNA or messenger RNA from pure cultures or the environment (Figure 1.b), labeling it with specific fluorescent molecules, and hybridizing it to target DNA spotted on the glass slide. The resulting image of

fluorescent spots is visualized by confocal laser scanning and digitized for quantitative analysis.

The most widely distributed research application of DNA microarrays is gene expression profiling, i.e. the identification of changes in mRNA expression of strains exposed to a particular substrate, for example a specific xenobiotic.

Microarrays have been already evaluated to study the physiology of environmental pure cultures. In this respect, Schut et al. (2001) constructed a DNA microarray with probes targeting 271 open reading frames from the genome sequence of the hyperthermophile *Pyrococcus furiosus*. When the strain was grown with elemental sulfur (S°), two previously uncharacterized operons were identified and their products were proposed to be part of a novel S°-reducing, membrane-associated, iron-sulfur cluster-containing complex. DNA microarrays were also used to study changes in mRNA expression levels in *Bacillus subtilis* grown under anaerobic conditions (Ye et al. 2000). Transcriptional activities of more than one hundred genes affected by the oxygen-limiting conditions were identified (Ye et al. 2000).

In addition to pure cultures, microarrays have also been used for physiological studies of environmental samples. Wu et al. (2001) detected genes involved in nitrogen cycling (*nirS*) with only 1 ng of labelled genomic DNA of a *P. stutzeri* strain and with 25 ng of bulk community DNA extracted from soil samples. Monitoring of catabolic genes involved in the degradation of xenobiotics was realised by Dennis et al. (2003). The authors designed a microarray containing probes targeting genes involved in the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). Induction of these genes was observed in the presence of 2,4-D with a 2,4-D degrading bacterium *Ralstonia eutropha* growing alone or diluted into a constructed mixed microbial community.

In microarray technology, sequence information is needed to design probes. However, this approach cannot be applied for discovering new catabolic genes for which no sequences are available in databases. Fortunately, the knowledge of the entire sequence is not necessary for the construction of microarrays; PCR products of a random genomic library constructed from a microorganism of interest may be spotted on glass slides. In response to a toxic substrate, one can expect differential gene expression at the transcript level. This will be reflected by differential hybridization patterns in the presence or the absence of the toxic pollutant.

Afterwards, clones of the library associated with differentially hybridized probes can be picked up for sequencing. This methodology was used by Parro and Moreno-Paz (2003) and allowed them to identify most of the genes involved in nitrogen fixation in *Leptospirillum ferrooxidans*. However, in the work of Parro and Moreno-Paz (2003), cultivation was necessary for construction of the genomic library. Alternatively, metagenomes may be utilized as shown by Sebat and coworkers (2003). These authors derived a cosmid library from a microcosm of groundwater and used this library as probes for microarrays. Afterwards, they hybridized the microarrays with cDNA of individual strains isolated from the microcosm and cDNA of the microcosm itself. By comparing the hybridization profiles of the microcosm with isolated strains, they could identify clones in the library corresponding to uncultured members of the microcosm. Sequencing of these clones revealed ORF's assigned to functions that have potential ecological importance, including hydrogen oxidation, nitrate reduction and transposition (Sebat et al. 2003).

Hence, a random genomic library approach associated with microarrays presents a high potential for the discovery of novel genes and operons. This can be extremely valuable because it makes possible not only to understand the biology of a microorganism, but also to get a more precise knowledge of biodegradation processes of xenobiotics for applications in pollution control and prevention under field conditions.

A2.6. Challenges of the microarray technology

One challenge in applying microarrays to environmental samples lies in the presence of humic matter, organic contaminants, and metals which may interfere with RNA and DNA hybridisation (Zhou and Thompson 2002). In addition to these contaminants, extraction of undegraded mRNA is an additional problem (Burgmann et al. 2003). Another challenge concerns the specificity of the method. In other words, sufficient discrimination between probes has to be achieved. Nevertheless, a discrimination level of one base-pair between probes could be achieved by imposing a temperature gradient and recording signal intensities (El Fantroussi et al. 2003; Urakawa et al. 2003). In this way, specific melting profiles could be generated for each probe. Likewise, one should keep in mind that the sensitivity of the method is 100 to 10,000-fold less than the PCR (Zhou and Thompson 2002) and this might be problematic for sequences of poor abundance. Last but not least, a promising perspective of microarrays lies in the possibility of determining, in complex environments, the relative abundance of a microorganism bearing a specific functional gene. Wu and coworkers (2001) observed a linear relationship between signal intensity and target DNA concentration over a range of 1 to 100 ng of genomic DNA from both pure cultures and mixed communities. However, as pointed out before, specificity is a key issue since it is imperative to distinguish differences in hybridization signals due to population abundance from those of sequence divergence.

A2.7. When 'function-driven' and 'sequence-driven' approaches meet

Interestingly, 'functional' and 'sequence-driven' approaches can complement each other. In the case of metagenomic libraries, sequencing clones with interesting functional properties can reveal a sequence that can be used to infer the phylogenetic affiliation of the organism from which the DNA was isolated, and sequencing of clones carrying a rRNA sequence in a big fragment can lead to functional information about the microorganism from which the fragment originated (Beja et al. 2000; Liles et al. 2003; Quaiser et al. 2003).

As environmental systems generally contain a high diversity of bacteria, the use of labeled xenobiotics can provide information of active bacteria among a complex environmental system. For example, by adding a radiolabeled substrate (^{13}C, ^{14}C and/or ^{15}N), active bacteria will incorporate labeled atoms into their DNA, making it denser than non-labeled DNA. By centrifugation, it is possible to separate labeled from non-labeled DNA (Radajewski et al. 2000) and therefore distinguish bacteria involved in the catabolic process or not. This labeled DNA can be used afterwards to construct metagenomic libraries. By enriching the genetic material of active microorganisms, this method is an excellent strategy for improving the likelihood of finding clones carrying catabolic genes.

Furthermore, if microarrays are dependent of genetic sequences, they can also accommodate a functional approach. For example, after incubation with ^{14}C-bicarbonate, Adamczyk et al. (2003) were able to identify microorganisms with CO_2 fixation activity in samples of nitrifying activated-sludge. Active microorganisms incorporated radioactive carbon into their RNA. After extraction of total rRNA and hybridization to a chip containing probes targeting ammonia-oxidizing bacteria, it was possible to scan microarray probes for radioactivity and identify the community structure of ammonia-oxidizing bacteria.

In summary, metagenomic libraries open access to the world of uncultivated microorganisms and their undescribed catabolic genes for the degradation of xenobiotics. Microarrays are also useful for discovering new catabolic genes. In addition, it provides the opportunity of easily monitoring catabolic genes. In both approaches, the use of radiolabeled molecules could improve the recovery and identification of microorganisms involved in the biodegradation of

xenobiotics. Despite technical challenges linked to the application of metagenomic libraries and microarrays, these methods present an exciting potential for unraveling the scientific bases of microbial degradation of xenobiotics.

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