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MINI-REVIEW

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

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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, the catabolic degradation pathways of xenobiotics and the 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 with traditional molecular techniques dependent on the isolation of pure cultures in the laboratory, microarrays and metagenomics allow specific environmental questions to be answered by exploring and using the phenomenal resources of uncultivable and uncharacterized micro-organisms. This paper reviews the current potential of microarrays and metagenomics to investigate the genetic diversity of environmentally relevant micro-organisms and identify new functional genes involved in the catabolism of xenobiotics.

Introduction

Xenobiotics are defined as compounds that are foreign to a living organism. These molecules are not easily recognized by existing degradative enzymes and tend to accumulate in soils and water. Among the 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, micro-organisms are the best candidates among all living organisms to funnel xenobiotic compounds into natural biogeochemical cycles. Indeed, more and more micro-organisms are being described as able to degrade these anthropogenic molecules. However, some xenobiotics have been shown to be unusually recalcitrant, i.e., micro-organisms 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 *Pseudomonas* and *Clostridia*) are 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. Indeed, the large majority of the earth's micro-organisms remain 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 micro-organisms (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 past 20 years, different molecular methods independent of cultivation have been developed to explore

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the diversity of micro-organisms, cultivable or not, in natural environments. Most of these methods are based on PCR amplification and subsequent analysis of bacterial rRNA genes by sequencing and fingerprint methods [clone libraries, RFLP, denaturing gradient gel electrophoresis (DGGE), etc.]. The discovery of many new bacterial lineages and 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 micro-organisms 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 et al. (2004): using a cultivation-independent molecular approach, he found thousands of new bacterial species and more than one million new protein-coding genes in 200 l of Sargasso seawater.

Powerful molecular methods have been recently developed to explore simultaneously the astonishing taxonomic and functional variety of environmental micro-organisms, like metagenomic libraries and microarrays. In the first methodology, libraries from the environmental metagenome are constructed and clones are screened either 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 onto a chip that can then be used to monitor the response of micro-organisms 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.

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 (Fig. 1a). Then, genomic DNA is enzymatically or mechanically fragmented. Fragments can be separated on the basis of their size by pulsed field gel electrophoresis. In this way, fragments of an appropriate size can be isolated from the gel and inserted into host cells by cloning vectors [bacteriophage lambda, cosmid, fosmid or bacterial artificial chromosome (BAC) vectors]. BAC vectors are especially efficient in maintaining stably large DNA inserts (up to 300 kb) in low copy numbers in the host cells (1–2 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-source and energy-source. Consequently, growth measurements could identify clones bearing catabolic genes.

The main limitation of this method may be the low recovery of active clones, because the function is not always expressed in the host cell, especially when not all the genes required for the function are 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. For example, genes involved in the biodegradation or biotransformation of nitroaromatic compounds identified so far are clustered in fragments of 5–27 kb (Table 1). This table also illustrates the fact that most of the catabolic genes known to date were isolated from culturable micro-organisms, after the 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

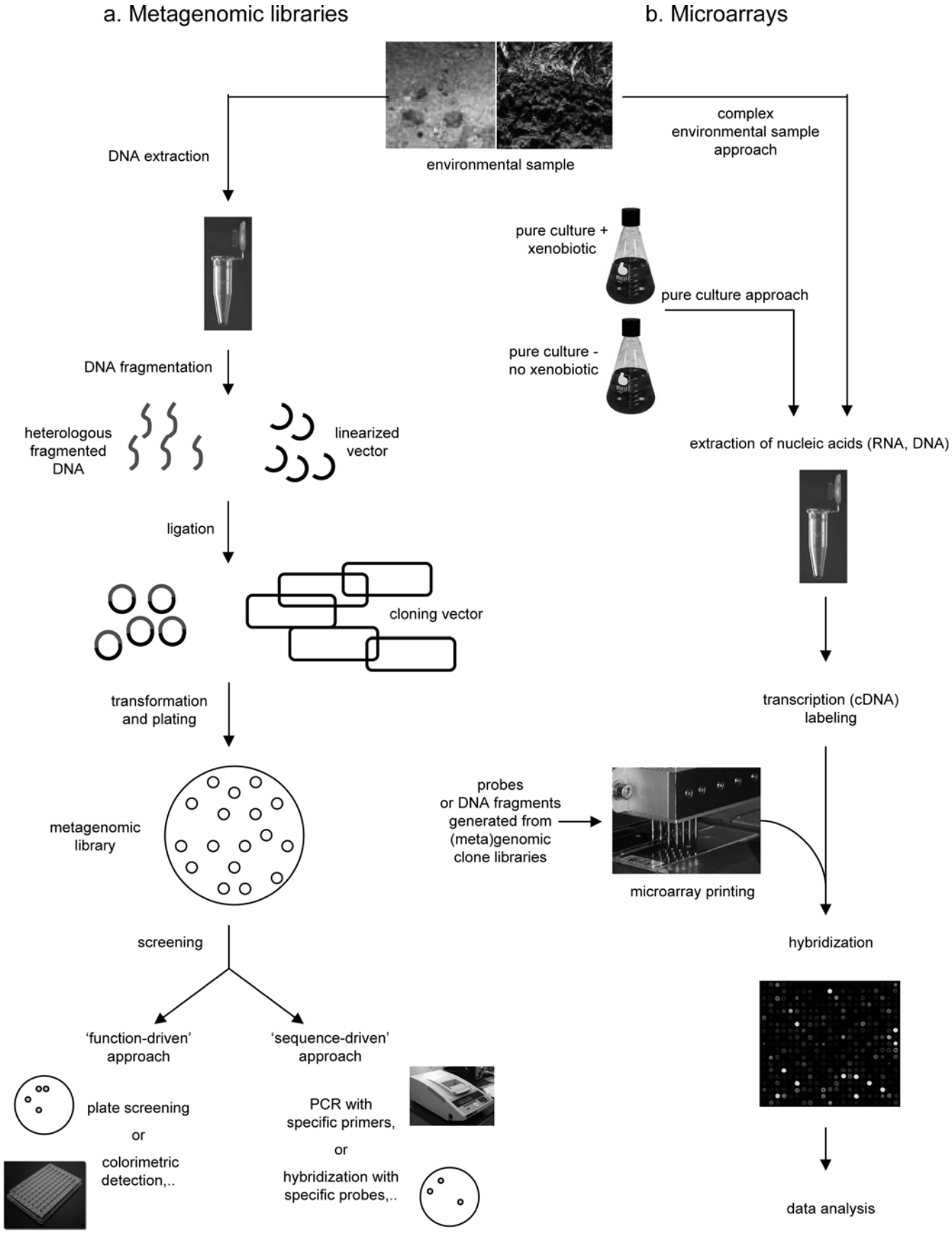

Fig. 1 **a** Construction and analysis of metagenomic libraries from environmental samples. **b** Construction of microarrays and hybridization with complex environmental samples or pure cultures

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

Target compound	Name	Target metabolite in pathway	Function	Size	Micro-organism of origin	Mode of isolation	References
4-Nitrotoluene	*ntnMA*	4-Nitrotoluene	Monooxygenase	14.8 kb	*Pseudomonas* sp. TW3	Genomic library screened with designed probes; activities confirmed by cloning and expression in *Escherichia coli*	James and Williams (1998); James et al. (2000)
	ntnD	4-Nitrobenzyl alcohol	Nitrobenzyl alcohol dehydrogenase				
	ntnC	4-Nitrobenzaldehyde	Nitrobenzaldehyde dehydrogenase				
	pnbA	4-Nitrobenzoate	Nitrobenzoate reductase	6 kb	*Pseudomonas* sp. TW3	Genomic library expressed in *P. putida* PaW340 and screening for metabolic activities	Hughes and Williams (2001)
	pnbB	4-Hydroxylaminobenzoate	Hydroxylaminobenzoate lyase				
2-Nitrotoluene	*ntdAa*	2-Nitrotoluene	Reductase	4.9 kb	*Pseudomonas* sp. JS42	Genomic library expressed in *E. coli* and screening for metabolic activities	Parales et al. (1996)
	ntdAb		Ferrodoxin				
	ntdAc		Iron–sulfur protein α				
	ntdAd		Iron–sulfur protein β				
2,4-Dinitrotoluene	*dntA*	2,4-Dinitrotoluene	Dioxygenase	27 kb	*Burkholderia cepacia* R34	Genomic library expressed in *E. coli* and screening for metabolic activities	Johnson et al. (2002)
	dntB	4-Methyl-5-nitrocatechol	Monooxygenase				
	dntD	2,4,5-Trihydroxytoluene	Dioxygenase				
	dntG	2,4-Dihydroxy-5-methyl-6-oxo-2,4-hexadecanoic acid	Isomerase/hydrolase				
	dntE	Methylmalonic acid semialdehyde	Dehydrogenase				
2,4,6-Trinitrophenol	*npdI*	2,4,6-Trinitrophenol	Hydride transferase	12.5 kb	*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)
	npdG	Hydride–Meisenheimer complex of 2,4,6-trinitrophenol	Hydride transferase				
	npdC	2,4,6-Trinitrophenol and its hydride–Meisenheimer	NADPH reductase				
2,4,6-Trinitrotoluene	*xenB*	2,4,6-Trinitrotoluene	Reductase	1.05 kb	*P. fluorescens* I-C	Genomic library expressed in *E. coli* and screening for metabolic activities	Blehert et al. (1999)

consortium in this polluted soil was demonstrated by DGGE (Eyers et al. 2004). Therefore, metagenomic libraries are particularly promising for finding denitration genes, compared with 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 hybridization probes and PCR primers, whose design is based on the information available in databases. Such hybridization probes may use, in the case of denitration of 2,4,6-trinitrophenol or other electron-deficient aromatics, the *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 the 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).

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 nonetheless can be responsible for a 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 thousands of clones, which can be laborious and time-consuming. High-throughput equipment is now available to facilitate colony-picking, inoculation in microtitre plates and screening numerous clones at the same time. 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 in biodegradation pathways. Communities enriched naturally by long-term exposure to high concentrations of xenobiotics may host the genes of interest at a high frequency. Pools of genes of interest might also be enriched by classic 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 is the case for polychlorinated biphenyl compounds (Abraham et al. 2002). In this case, it is not possible to isolate a contiguous piece of DNA containing 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 both relatively insensitive to the toxic xenobiotic and unable to catabolize it in the absence of the vector.

Microarrays

The genetic information accumulated by major research programs, such as the sequencing of the human genome and the genomes of hundreds of other organisms, provides 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, etc.), are printed at high density on a substrate.

Compared with 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 are spotted in a precise pattern. There are two principal microarray types based on the nature of the target (DNA, e.g., cDNA, PCR products, oligonucleotides or plasmids, or RNA) and on the method of spotting (mechanical microspotting or photolithography). In the first method, purified DNA is spotted onto the membrane or coated glass slide. Although DNA will stick to glass, aminosilane-coated and poly-L-lysine (PLL)-coated slides are predominantly used. Silanized slides ($RSiX_3$, where X is typically alkoxy) attach the 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 the microarrays, the next step involves extracting DNA or messenger RNA from pure cultures or the environment (Fig. 1b), labelling 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 is 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 evaluated to study the physiology of pure environmental cultures. In this respect, Schut et al. (2001) constructed a DNA microarray with probes targeting 271 open reading frames (ORFs) 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 have also been used to study changes in mRNA expression levels in *Bacillus subtilis* grown under anaerobic conditions (Ye et al. 2000). Transcriptional activities of more than 100 genes affected by the oxygen-limiting conditions were identified (Ye et al. 2000).

In addition to pure cultures, microarrays have been used for physiological studies of environmental samples. Wu et al. (2001) detected genes involved in nitrogen-cycling (*nirS*), using only 1 ng of labelled genomic DNA of a *Pseudomonas stutzeri* strain and 25 ng of bulk community DNA extracted from soil samples. Monitoring of catabolic genes involved in the degradation of xenobiotics was realized 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 the 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, knowledge of the entire sequence is not necessary for the construction of microarrays; and PCR products of a random genomic library constructed from a micro-organism of interest may be used. In response to a toxic substrate, one can expect differential gene expression at the transcript level. This is reflected by differential hybridization patterns in the presence or 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 et al. (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 ORFs 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 is then possible not only to understand the biology of a micro-organism, but also to get a more precise knowledge of biodegradation processes of xenobiotics for applications in pollution control and prevention under field conditions.

The challenges of 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 hybridization (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 1 bp 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 that of PCR (Zhou and Thompson 2002) and this might be problematic for sequences of poor abundance. Last but not least, a promising perspective for microarrays lies in the possibility of determining, in complex environments, the relative abundance of a micro-organism bearing a specific functional gene. Wu et al. (2001) observed a linear relationship between signal intensity and target DNA concentration over a range of 1–100 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 due to sequence divergence.

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 may reveal a sequence that can be used to infer the phylogenetic affiliation of the organism from which the DNA was isolated; and sequencing clones that carry a rRNA sequence in a big fragment can lead to functional information about the micro-organism 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 labelled xenobiotics can provide information about active bacteria within a complex environmental system. For example, adding a radio-labelled substrate (^{13}C, ^{14}C, ^{15}N) allows active bacteria to incorporate labelled atoms into their DNA, making it denser than non-labelled DNA. By centrifugation, it is possible to separate labelled from non-labelled DNA (Radajewski et al. 2000) and therefore distinguish the bacteria involved in the catabolic process from those which are not. This labelled DNA can be used afterwards to construct metagenomic libraries. By enriching the genetic material of active micro-organisms, this method is an excellent strategy for improving the likelihood of finding clones carrying catabolic genes.

Furthermore, if microarrays are dependent on 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 micro-organisms with CO_2-fixing activity in samples of nitrifying activated sludge because the active micro-organisms incorporated the 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 micro-organisms 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 radio-labelled molecules could improve the recovery and identification of micro-organisms 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 unravelling the scientific basis of microbial degradation of xenobiotics.

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