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MINI-REVIEW

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Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacteria and their catabolic genes in bioremediation

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Abstract The pollution of soil and water with xenobiotics is widespread in the environment and is creating major health problems. The utilization of microorganisms to clean up xenobiotics from a polluted environment represents a potential solution to such environmental problems. Recent developments in molecular-biology-based techniques have led to rapid and accurate strategies for monitoring, discovery and identification of novel bacteria and their catabolic genes involved in the degradation of xenobiotics. Application of these techniques to bioremediation has also improved our understanding of the composition, phylogeny, and physiology of metabolically active members of the microbial community in the environment. This review provides an overview of recent developments in molecular-biology-based techniques and their application in bioremediation of xenobiotics.

Introduction

The pollution of soil and water with xenobiotics is a problem of increasing magnitude (Moriarty 1988). In situ clean-up may include bioremediation (Madsen 1991, Madsen et al. 1991), which can be defined as: (1) a method of monitoring the natural progress of degradation to ensure that the contaminant decreases with sampling time (bioattenuation), (2) the intentional stimulation of resident xenobiotic-degrading bacteria by electron acceptors, water, nutrient addition, or electron donors (biostimulation), or (3) the addition of laboratory-grown bacteria that have appropriate degradative abilities (bioaugmentation).

Since bacteria are usually the agents in most bioremediation processes, the evaluation of a polluted site before bioremediation often involves detection and enumeration of the quantity and activity of xenobiotic-degrading bacteria. Such studies require enumeration techniques that produce accurate results rapidly and safely (Bogardt and Hemmingsen 1992).

Enumeration and monitoring of xenobiotic-degrading bacterial populations in contaminated environments using traditional microbiological methods can take an inordinate length of time, and often underestimates numbers as a result of our inability to cultivate the majority of soil organisms (Lloyd-Jones et al. 1999). Molecular approaches are now being used to characterize the nucleic acids of microorganisms contained in the microbial community from environmental samples (Fig. 1). The major benefit of these molecular analyses is the ability to study microbial communities without culturing of bacteria and fungi, whereas analyses using incubation in the laboratory (classic microbiology) are indirect and produce artificial changes in the microbial community structure and metabolic activity. In addition, direct molecular methods preserve the in situ metabolic status and microbial community composition, because samples are frozen immediately after acquisition. Also, direct extraction of nucleic acids from environmental samples accounts for the very large proportion of microorganisms (90–99.9%) that are not readily cultured in the laboratory, but that may be responsible for the majority of the biodegradative activity of interest (Brockman 1995). When combined with classic microbiological methods, these molecular biological methods will provide us with a more comprehensive interpretation of the in situ microbial community and its response to both engineered bioremediation and natural attenuation processes (Brockman 1995).

Bioaugmentation is useful when effective pollutant-degrading populations are not present at a polluted site (Watanabe et al. 1998). Successful bioaugmentation re-

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Fig. 1 Molecular approaches for detection and identification of xenobiotic-degrading bacteria and their catabolic genes from environmental samples (adapted from Muyzer and Smalla 1998)

quires that strains suitable for each polluted site be selected. For example, at a polluted site, the strain introduced must be able to survive and retain high enough catabolic activity to degrade the pollutant. In this respect, it is desirable to establish evaluation methods for monitoring and containment of the bioaugmented bacteria.

In this review we summarize recent developments in molecular-biology-based techniques for enumeration of environmental bacteria and bioaugmented bacteria in bioremediation of xenobiotic contamination.

In situ analysis of microbial community and activity in bioremediation

DNA-based methods

A probe DNA may detect genes or gene sequences in total DNA isolated and purified from environmental samples by a variety of methods. DNA hybridization techniques, using labeled DNA as a specific probe have been used in the past for identification of specific microorganisms in environmental samples (Atlas 1992; Sayler and Layton 1990). Although these techniques are still useful for monitoring a specific genome in nature, they have some limitations. Colony hybridization can only be used for detection of culturable cells, and slot blot and Southern blot hybridization methods are not adequately sensitive for the detection of low cell numbers. On the other hand, greater sensitivity of detection, without reliance on cultivation, can be obtained using PCR (Jansson 1995).

One of the earliest studies to use direct hybridization techniques for monitoring xenobiotic degraders, monitored the TOL (for toluene degradation) and NAH (for naphthalene degradation) plasmids in soil microcosms (Sayler et al. 1985). Colonies were hybridized with entire plasmids as probes to quantify the cells containing these catabolic plasmids. A positive correlation was observed between plasmid concentrations and the rates of mineralization. Exposure to aromatic substrates caused an increase in plasmid levels (Sayler et al. 1985). Similar tech-

niques were used recently to monitor the *xylE* and *ndoB* genes involved in creosote degradation in soil microcosms (Hosein at al. 1997). Standard Southern blot hybridization has been used to monitor bacterial populations of naphthalene-degraders in seeded microcosms induced with salicylate (Ogunseitan et al. 1991). In this study, probes specific for the *nah* operon were used to determine the naphthalene-degradation potential of the microbial population. Dot-blot hybridizations with isolated polychlorinated biphenyl (PCB) catabolic genes have been used to measure the level of PCB-degrading organisms in soil microbial communities (Walia et al. 1990).

Molecular probing has been used in conjunction with traditional most-probable-number (MPN) techniques in several studies. A combination of MPN and colony hybridization was used to monitor the microbial community of a flow-through lake microcosm seeded with a chlorobenzoate-degrading *Alcaligenes* strain (Fulthorpe and Wyndham 1989). This study revealed a correlation between the size and activity of a specific catabolic population during exposure to various concentrations of 3-chlorobenzoate. In another study, Southern hybridization with *tfdA* and *tfdB* gene probes was used to measure the 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading populations in field soils (Holben et al. 1992). It was shown that amendment of the soil with 2,4-D increased the level of hybridization and that these changes agreed with MPN analyses.

RNA-based methods

One disadvantage of DNA-based methods is that they do not distinguish between living and dead organisms, which limits their use for monitoring purposes. The mRNA level may provide a valuable estimate of gene expression and/or cell viability under different environmental conditions (Fleming et al. 1993). Retrieved mRNA transcripts can be used to compare the expression level of individual members of gene families in the environment. Thus, when properly applied to field samples, mRNA-based methods may be useful in determining the relationships between the environmental conditions prevailing in a microbial habitat and particular in situ activities of native microorganisms (Wilson et al. 1999). Extraction of RNA instead of DNA, followed by reverse-transcription-PCR (RT-PCR), gives a picture of the metabolically active microorganisms in the system (Nogales et al. 1999; Weller and Ward 1989). RT-PCR adds an additional twist to the PCR technique. Before PCR amplification, the DNA in a sample is destroyed with DNase. Reverse transcriptase and random primers (usually hexamers) are added to the reaction mixture, and the RNA in the sample – including both mRNA and rRNA – is transcribed into DNA. PCR is then used to amplify the specific sequences of interest. RT-PCR gives us the ability to detect and quantify the expression of individual structural genes. In a recent study, the fate of phenol-degrading *Pseudomonas* was monitored in bioaugmented sequencing batch reactors fed with synthetic petrochemical wastewater by using PCR amplification of the *dmpN* gene (Selvaratnam et al. 1995, 1997). In addition, RT-PCR was used to measure the level of transcription of the *dmpN* gene. Thus, not only was the presence of organisms capable of phenol degradation detected, but the specific catabolic activity of interest was also measured. A positive correlation was observed between the level of transcription, phenol degradation, and periods of aeration. In a similar study, transcription of the *tfdB* genes was measured by RT-PCR in activated-sludge bioreactors augmented with a 3-chlorobenzoate-degrading *Pseudomonas* (Selvaratnam et al. 1997), and the expression of a chlorocatechol 1,2-dioxygenase gene (*tcbC*) in river sediment was measured by RT-PCR (Meckenstock et al. 1998). Similarly, with this approach Wilson et al. (1999) isolated and characterized in situ transcribed mRNA from groundwater microorganisms catabolizing naphthalene at a coal-tar-waste-contaminated site using degenerate primer sets. They found two major groups related to the dioxygenase genes *ndoB* and *dntAc*, previously cloned from *Pseudomonas putida* NCIB 9816-4 and *Burkholderia* sp. strain DNT, respectively. Furthermore, the sequencing of the cloned RT-PCR amplification product of 16S rRNA generated from total RNA extracts has been used to identify presumptive metabolically active members of a bacterial community in soil highly polluted with PCB (Nogales et al. 1999).

Differential display (DD), an RNA-based technique that is widely used almost exclusively for eukaryotic gene expression, has been recently optimized to assess bacterial rRNA diversity (Yakimov et al. 2001). Double-stranded cDNAs of rRNAs were synthesized without a forward primer, digested with endonuclease, and ligated with a double-stranded adapter. The fragments obtained were then amplified using an adapter-specific extended primer and a 16S rDNA universal primer pair, and displayed by electrophoresis on a polyacrylamide gel (Yakimov et al. 2001). In addition, the DD technique has been optimized and used to directly clone actively expressed genes from soil-extracted RNA (Fleming et al. 1998). Using this approach, Fleming et al. (2001) successfully cloned a novel salicylate-inducible naphthalene dioxygenase from *Burkholderia cepacia* (Fleming et al. 1998), and identified the bacterial members of a 2,4,5-trinitrophenoxyacetic acid-degrading consortium.

Nucleic acid extraction and purification methods for environmental samples

Nucleic acid isolation from an environmental sample is the most important step in examining the microbial community and catabolic gene diversity. Procedures for DNA isolation from soil and sediment were first developed in the 1980s, and can be divided into two general categories: (1) direct cell lysis followed by DNA purification steps, and (2) bacterial isolation followed by cell lysis and DNA purification. Since that time, these methods have been

continually modified and improved. The methods for fractionation of bacteria as a preliminary step (Bakken and Lindahl 1995; Torsvik et al. 1995) and for direct extraction (Saano et al. 1995; Trevors and van Elsas 1995) have recently been compiled. In general, DNA isolation methods are moving from the use of large samples and laborious purification procedures towards the processing of small samples in microcentrifuge tubes (Dijkmans et al. 1993; More et al. 1994). In addition, methods for efficient bacterial cell lysis have been evaluated and improved. Bead-mill homogenization has been shown to lyse a higher percentage of cells (without excessive DNA fragmentation) than freeze-thaw lysis although 'soft lysis' by freezing and thawing is useful for obtaining high molecular weight DNA (Erb and Wagner-Dobler 1993; Miller et al. 1999). The efficiency of cell lysis and DNA extraction varies with sample type and DNA extraction procedure (Erb and Wagner-Dobler 1993; Frostegard et al. 1999; Miller et al. 1999). Therefore, in order to obtain accurate and reproducible results, the variation in the efficiency of cell lysis and DNA extraction must be taken into account. Co-extraction with standard DNA has been used to overcome the bias in extraction of DNA from Baltic Sea sediment samples (Moller and Jansson 1997). However, the methods reported do not overcome the variability in the efficiency of cell lysis. In contrast to extraction of DNA, extraction of mRNA from environmental samples is quite difficult and is further hampered by the half-lives of prokaryotic mRNA being very short.

An ideal procedure for recovering nucleic acids from environmental samples has recently been summarized by Hurt et al. (2001). They state that an ideal procedure should meet several criteria: (1) the nucleic acid recovery efficiency should be high and not biased so that the final nucleic acids are representative of the total nucleic acids within the naturally occurring microbial community; (2) the RNA and DNA fragments should be as large as possible so that molecular studies, such as community gene library construction and gene cloning, can be carried out; (3) the RNA and DNA should be of sufficient purity for reliable enzyme digestion, hybridization, reverse transcription, and PCR amplification; (4) the RNA and DNA should be extracted simultaneously from the same sample so that direct comparative studies can be performed (this will also be particularly important for analyzing samples of small size); (5) the extraction and purification protocol should be kept simple as much as possible so that the whole recovery process is rapid and inexpensive; and (6) the extraction and purification protocol should be robust and reliable, as demonstrated with many diverse environmental samples. However, none of the previously mentioned nucleic acid extraction methods have been evaluated and optimized based on all the above important criteria.

Genetic fingerprinting techniques

Genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community. Recently, several fingerprinting techniques have been developed and used in microbial ecology studies such as bioremediation.

The separation of, or detection of small differences in, specific DNA sequences can give important information about the community structure and the diversity of microbes containing a critical gene. Generally, these techniques are coupled to a PCR reaction to amplify sequences that are not abundant. PCR-amplified products can be examined by using techniques that detect single substitutions in the nucleotide sequence (Scheegurt-Mark and Kulpa-Chaler 1998). These techniques are important in separating and identifying PCR-amplified products that might have the same size but slightly different nucleotide sequences. For example, the amplified portions of *nahAc* genes from a mixed microbial population might be of similar size when amplified with a particular set of *nahAc*-specific degenerate primers, but have small differences within the PCR-amplified products at the nucleotide level. One way of detecting these differences is to digest the PCR-amplified product with restriction endonucleases and examine the pattern of restriction fragments. The PCR-amplified product can be end-labeled or uniformly labeled for this technique.

In one study, natural sediments were tested for the presence of *nahAc* gene sequences by using PCR (Herrick et al. 1993). Polymorphisms in this gene sequence were detected by restricting the PCR-amplified products. In another study, PCR amplification of *bphC* genes by using total DNA extracted from natural soils as template allowed further investigation of the PCB degradation pathway (Erb and Wagner-Dobler 1993). No restriction polymorphisms were observed in the PCR-amplified products, suggesting limited biodiversity in this PCB-degrading population. Contaminated soils gave positive results, whereas pristine lake sediments did not contain appreciable amounts of the *bphC* gene.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry (MALDI-TOF-MS) has been developed as a rapid and sensitive method for analyzing the restriction fragments of PCR-amplified products (Taranenko et al. 2002). A mass spectrum can be obtained in less than 1 min.

Another advanced method, terminal restriction fragment length polymorphism (T-RFLP) analysis, measures the size polymorphism of terminal restriction fragments from a PCR-amplified marker. It combines at least three technologies, including comparative genomics/RFLP, PCR, and electrophoresis. Comparative genomics provides the necessary insight to allow design of primers for amplification of the target product, and PCR amplifies the signal from a high background of unrelated markers. Subsequent digestion with selected restriction endonucleases produces terminal fragments appropriate for sizing on high resolution (±1-base) sequencing gels. The latter step is conveniently performed on automated systems such as polyacrylamide gel or capillary electrophoresis systems that provide digital output. The use of a fluorescently tagged primer limits the analysis to only

the terminal fragments of the digestion. Because size markers bearing a different fluorophore from the samples can be included in every lane, the sizing is extremely accurate (Marsh 1999).

Denaturing gradient gel electrophoresis (DGGE) and its cousin TGGE (thermal-GGE) is a method by which fragments of DNA of the same length but different sequence can be resolved electrophoretically (Muyzer 1999). Separation is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of a denaturing reagent (a mixture of formamide and urea) or a linear temperature gradient (Muyzer et al. 1993). As the duplex DNA fragments are subjected to electrophoresis, partial melting occurs at denaturant concentrations specific for various nucleotide sequences. An excellent study by Watanabe and coworkers (Watanabe et al. 1998) used a combination of molecular-biological and microbiological methods to detect and characterize the dominant phenol-degrading bacteria in activated sludge. TGGE analysis of PCR products of 16S rDNA and of the gene encoding phenol hydroxylase (LmPH) showed a few dominant bacterial populations after a 20-day incubation with phenol as a carbon source. Comparison of sequences of different bacterial isolates and excised TGGE bands revealed two dominant bacterial strains responsible for the phenol degradation (Watanabe et al. 1998).

Watts et al. (2001) recently reported a comparative analysis of PCB-dechlorinating communities in enrichment cultures using three different molecular screening techniques, namely, amplified ribosomal DNA restriction analysis (ARDRA), DGGE, and T-RFLP. They found that the methods have different biases, which were apparent from discrepancies in the relative clone frequencies (ARDRA), band intensities (DGGE) or peak heights (T-RFLP) from the same enrichment culture. However, all of these methods were useful for qualitative analysis and could identify the same organism (Watts et al. 2001). Overall, in community fingerprinting and preliminary identification, DGGE proved to be the most rapid and effective tool for monitoring microorganisms within a highly enriched culture. T-RLFP results corroborated DGGE fingerprint analysis, but the identification of the bacteria detected required the additional step of creating a gene library. ARDRA provided an in-depth analysis of the community and this technique detected slight intra-species sequence variation in 16S rDNA (Watts et al. 2001).

Another such approach takes advantage of sequence-dependent conformational differences between re-annealed single-stranded products (SSCP), which also result in changes in electrophoretic mobility; DNA fragments are separated on a sequencing gel under non-denaturing conditions based on their secondary structures (Schiwieger and Tebbe 1998).

Recently, a method using denaturing high performance liquid chromatography (DHPLC) was developed that can detect single base-pair mutations within a specific sequence (Taliani et al. 2001). This is a rapid, sensitive and accurate method of detecting sequence variation. However, this method has not yet been used for analyzing the diversity of specific sequences from environmental samples. DHPLC could be a useful, rapid and sensitive method for ecological study in bioremediation.

Discovery of novel catabolic genes involved in xenobiotic degradation

There are two different approaches to investigate the diversity of catabolic genes in environmental samples: culture-dependent and culture-independent methods. In culture-dependent methods, bacteria are isolated from environmental samples with culture medium. Nucleic acid is then extracted from the bacterial culture. In contrast, culture-independent methods employ direct extraction of nucleic acids from environmental samples (Lloyd-Jones et al. 1999; Okuta et al. 1998; Watanabe et al. 1998). The description of catabolic gene diversity by culture-independent molecular biological methods often involves the amplification of DNA or cDNA from RNA extracted from environmental samples by PCR, and the subsequent analysis of the diversity of amplified molecules (community fingerprinting). Alternatively, the amplified products may be cloned and sequenced to identify and enumerate bacterial species present in the sample.

To date, more than 300 catabolic genes involved in catabolism of aromatic compounds have been cloned and identified from culturable bacteria. Several approaches, such as shotgun cloning by using indigo formation (Ensley et al. 1983; Goyal and Zylstra 1996), clearing zone formation (de Souza et al. 1995), or meta-cleavage activity (Sato et al. 1997) as screening methods for cloning; applying proteomics (two dimensional gel electrophoresis analysis) of xenobiotic-inducible proteins to obtain genetic information (Khan et al. 2001), transposon mutagenesis to obtain a defective mutant (Foght and Westlake 1996), transposon mutagenesis using a transposon-fused reporter gene (Bastiaens et al. 2001), applying a degenerate primer to generate a probe (Saito et al. 2000), and applying a short probe from a homologous gene (Moser and Stahl 2001), have been used to discover catabolic genes for aromatic compounds from various bacteria.

The emergence of methods using PCR to amplify catabolic sequences directly from environmental DNA samples now appears to offer an alternative technique to discover novel catabolic genes in nature. Most research focusing on analysis of the diversity of the catabolic genes in environmental samples has employed PCR amplification using a degenerate primer set (a primer set prepared from consensus or unique DNA sequence), and the separation of the resultant PCR products either by cloning or by gel electrophoresis (Allison et al. 1998; Hedlund et al. 1999; Lloyd-Jones et al. 1999; Watanabe et al. 1998; Wilson et al. 1999). To confirm that the proper gene has been PCR-amplified, it is necessary to sequence the product, after which the resultant information can be

Table 1 Molecular approaches for investigating the diversity and identification of catabolic genes involved in degradation of xenobiotics. *RT* Reverse transcription, *PCR* polymerase chain reaction, *DGGE* denaturing gradient gel electrophoresis, *RHD* ring hydroxylating dioxygenase, *PAH* polycyclic aromatic hydrocarbon

Target gene	Molecular approach	Source	Reference
nahAc	RT-PCR with degenerate primers	Groundwater (culture-independent)	Wilson et al. 1999
phnAc, nahAc, and glutathione-*S*-transferase	PCR with several primers	Soil samples (culture-independent)	Lloyd-Jones et al. 1999
Phenol hydroxylase (LmPH)	PCR-DGGE with degenerate primers	Activated sludge (culture-independent)	Watanabe et al. 1998
RHD	PCR with degenerate primers	Prestine- and aromatic hydrocarbon-contaminated soils (culture-independent)	Yeates et al. 2000
PAH dioxygenase	PCR with several primers	PAH soil bacteria (culture-dependent)	Lloyd-Jones et al. 1999
nahAc	PCR with degenerate primers	Marine sediment bacteria (culture-dependent)	Allison et al. 1998
nahAc	PCR with degenerate primers	Marine sediment bacteria (culture-dependent)	Hedlund et al. 1999
nah	PCR with degenerate primers	Soil bacteria (culture-dependent)	Hamann et al. 1999
tfdC	PCR with degenerate primers	Soil bacteria (culture-dependent)	Cavalca et al. 1999
PAH dioxygenase and catechol dioxygenase	PCR with degenerate primers	Wastewater and soil bacteria (culture-dependent)	Meyer et al. 1999
phnAc, *nahAc* and PAH dioxygenase	PCR with several degenerate primers	River water, sediment, and soil bacteria (culture-dependent)	Widada et al. 2002a
RHD	PCR-DGGE with degenerate primers	*Rhodococcus* sp. strain RHA1 (culture-dependent)	Kitagawa et al. 2001

used to reveal the diversity of the corresponding gene(s). Over the last few years, these molecular techniques have been systematically applied to the study of the diversity of aromatic-compound-degrading genes in environmental samples (Table 1).

Application of a degenerate primer set to isolate functional catabolic genes directly from environmental samples has been reported (Okuta et al. 1998). Fragments of catechol 2,3-dioxygenase (C23O) genes were isolated from environmental samples by PCR with degenerate primers, and the gene fragments were inserted into the corresponding region of the *nahH* gene, the structural gene for C23O encoded by the catabolic plasmid NAH7, to reconstruct functional hybrid genes reflecting the diversity in the natural gene pool. In this approach, the only information necessary is knowledge of the conserved amino acid sequences in the protein family from which the degenerate primers should be designed. This method is generally applicable, and may be useful in establishing a divergent hybrid gene library for any gene family (Okuta et al. 1998).

When degenerate primers cannot be used for amplification of DNA or RNA targets, PCR has limited application for investigating novel catabolic genes from culture collections or from environmental samples. Dennis and Zylstra (1998) have developed a new strategy for rapid analysis of genes for Gram-negative bacteria. For this purpose, they constructed a minitransposon containing an origin of replication in an *Escherichia coli* cell. These artificially derived transposons are called plasposons (Dennis and Zylstra 1998). Once a desired mutant has been constructed by transposition, the region around the insertion point can be rapidly cloned and sequenced. Mutagenesis with these plasposons can be used as an alternative tool to investigate novel catabolic genes from culture collections, although such approaches cannot be taken for environmental samples. The in vitro transposon mutagenesis by plasposon containing a reporter gene without a promoter will provide an alternative technique to search for desired xenobiotic-inducible promoters from environmental DNA samples.

Monitoring of bioaugmented microorganisms in bioremediation

Because different methods for enumeration of microorganisms in environmental samples sometimes provide different results, the method used must be chosen in accordance with the purpose of the study. Not all detection methods provide quantitative data; some only indicate the presence of an organism and others only detect cells in a particular physiological state (Jansson and Prosser 1997). Several molecular approaches have been developed to detect and quantify specific microorganisms (Table 2).

Table 2 Molecular approaches for detection and quantification of specific microorganisms in environmental samples (adapted from Jansson and Prosser 1997). *cPCR* Competitive PCR, *MPN-PCR* most probable number PCR, *RLD-PCR* replicative limiting dilution PCR

Identification method	Detection and quantification method	Cell type monitored
Fluorescent tags on rRNA probes	Microscopy Flow cytometry	Primary active cells
lux or *luc* gene	Luminometry/scintillation counting	Active cells
	Cell extract luminescence	Total cells with translated luciferase protein
	Luminescent colonies	Culturable luminescent cells
gfp gene	Fluorescent colonies	
	Microscopy	Culturable fluorescent cells
	Flow cytometry	Total cells, including starved
Specific DNA sequence	cPCR MPN-PCR, RLD-PCR	Total DNA (living and dead cell and free DNA)
	Slot/dot blot hybridization Colony hybridization	Culturable cells
Specific mRNA transcript	Competitive RT-PCR Slot/dot blot hybridization	Catabolic activity of cells
Other marker genes (e.g., *lacZY*, *gusA*, *xylE*, and antibiotic resistance genes)	Plate counts colony hybridization	Culturable marked cells and indigenous cells with marker phenotype
	Quantitative PCR Slot/dot blot hybridization	Total DNA (living and dead cells and free DNA)

Quantification by PCR/RT-PCR

PCR is now often used for sensitive detection of specific DNA in environmental samples. Sensitivity can be enhanced by combining PCR with DNA probes, by running two rounds of amplification using nested primers (Moller et al. 1994), or by using real-time detection systems (Widada et al. 2001). Detection limits vary for PCR amplification, but usually between 10^2 and 10^3 cells/g soil can routinely be detected by PCR amplification of specific DNA segments (Fleming et al. 1994b; Moller et al. 1994). Despite its sensitivity, until recently it has been difficult to use PCR quantitatively to calculate the number of organisms (gene copies) present in a sample. Three techniques have now been developed for quantification of DNA by PCR, namely: MPN-PCR, replicative limiting dilution-PCR (RLD-PCR), and competitive PCR (cPCR) (Chandler 1998).

MPN-PCR is carried out by running multiple PCR reactions of samples that have been serially diluted, and amplifying each dilution in triplicate. The number of positive reactions is compared to the published MPN tables for an estimation of the number of target DNA copies in the sample (Picard et al. 1996). In MPN-PCR, DNA extracts are serially diluted before PCR amplification and limits can be set on the number of genes in the sample by reference to known control dilutions.

RLD-PCR, an alternate quantitative PCR for environmental application, is based on RLD analysis and the pragmatic tradeoffs between analytical sensitivity and practical utility (Chandler 1998). This method has been used to detect and quantify specific biodegradative genes in aromatic-compound-contaminated soil. The catabolic genes *cdo*, *nahAc*, and *alkB* were used as target genes (Chandler 1998).

Quantitative cPCR is based on the incorporation of an internal standard in each PCR reaction. The internal standard (or competitor DNA) should be as similar to the target DNA as possible and be amplified with the same primer set, yet still be distinguishable from the target, for example, by size (Diviacco et al. 1992). A standard curve is constructed using a constant series of competitor DNA added to a dilution series of target DNA. The ratio of PCR-amplified DNA yield is then plotted versus initial target DNA concentration. This standard curve can be used for calculation of unknown target DNA concentrations in environmental samples. The competitive standard is added to the sample tube at the same concentration as used for preparation of the standard curve (Diviacco et al. 1992). Since both competitor and target DNAs are subjected to the same conditions that might inhibit the performance of DNA polymerase (such as humic acid or salt contaminants), the resulting PCR product ratio is still valid for interpolation of target copy number for the standard curve. Recently, Alvarez et al. (2000) have developed a simulation model for cPCR, which takes into account the decay in efficiency as a linear function of product yield. Their simulation data suggested that differences in amplification efficiency between target and standard templates induced biases in quantitative cPCR. Quantitative cPCR can only be used when both efficiencies are equal (Alvarez et al. 2000).

In bioremediation, quantitative PCR has been used to monitor and to determine the concentration of some catabolic genes from bioaugmented bacteria in environmental samples (Table 3). Recently, quantitative competitive RT-PCR has been used to quantify the mRNA of the *tcbC* of *Pseudomonas* sp. strain P51 (Meckenstock et al. 1998).

Molecular marker gene systems

In many laboratory biodegradation studies, bacterial cells that are metabolically capable of degrading/miner-

Table 3 PCR detection and quantification of introduced bacteria in bioremediation of xenobiotics

Bacteria	Target gene	Detection and quantification method	Reference
Desulfitobacterium frappieri strain PCP-1 (pentachlorophenol-degrader)	16 rRNA	Nested PCR	Levesque at al. 1997
Mycobacterium chlorophenolicum strain PCP-1 (pentachlorophenol-degrader)	16 rRNA	MPN-PCR	van Elsas et al. 1997
Sphingomonas chlorophenolica (pentachlorophenol-degrader)	16rRNA	Competitive PCR	van Elsas et al. 1998
Pseudomonas sp. strain B13 (chloroaromatic-degrader)	16 rRNA	Competitive PCR	Leser et al. 1995
Pseudomonas putida strain mx (toluene-degrader)	*xylE*	Competitive PCR	Hallier-Soulier et al. 1996
P. putida strain G7 (naphthalene-degrader)	*nahAc*	PCR-Southern blot	Herrick et al. 1993
P. putida strain mt2 (toluene-degrader)	*xylM*	Multiplex PCR-Southern blot	Knaebel and Crawford 1995
Psuedomonas oleovorans strain OCT (octane-degrader)	*alkB*		
Alcaligenes eutropus strain JMP134 [2,4-dichlorophenoxyacetic acid (2,4-D)-degrader]	*tfdA*		
P. putida ATCC 11172 (phenol-degrader)	*dmpN*	PCR and RT-PCR	Selvaratnam et al. 1997
Pseudomonas sp. strain P51 (trichlorobenzene-degrader)	*tbcAa, tbcC*	PCR	Tchelet et al. 1999
Pseudomonas sp. strain P51 (trichlorobenzene-degrader)	*tbcC*	Competitive RT-PCR	Meckenstock et al. 1998
Psuedomonas resinovorans strain CA10 (carbazole- and dibenzo-*p*-dioxin-degrader)	*carAa*	Real-time competitive PCR	Widada et al. 2001, 2002b

alizing a pollutant are added to contaminated environmental samples to determine the potential biodegradation of target compound(s). Assessment of environmental impact and risk associated with environmental release of augmented bacteria requires knowledge of their survival, persistence, activity, and dispersion within the environment. Detection methods that take advantage of unique and identifiable molecular markers are useful for enumerating and assessing the fate of microorganisms in bioremediation (Prosser 1994). The application of molecular techniques has provided much greater precision through the introduction of specific marker genes. Some of the requirements for marker systems include the ability to allow unambiguous identification of the marked strain within a large indigenous microbial community, its stable maintenance in the host cell, and adequate expression for detection (Lindow 1995).

Antibiotic resistance genes, such as the *nptII* gene encoding resistance to kanamycin, were the first genes to be employed as markers. Although they are still in use, these phenotypic marker genes are generally falling out of favor because of the small risk of contributing to the undesirable spread of antibiotic resistance in nature (Lindow 1995).

Genes encoding metabolic enzymes have also been used as non-selective markers. These include *xylE* (encoding catechol 2,3-oxygenase), *lacZY* (encoding β-galactosidase and lactose permease) and *gusA* (β-glucuronidase). The *xylE* gene product can be detected by the formation of a yellow catabolite (2-hydroxymuconic semialdehyde) from catechol. The enzymes encoded by *lacZ* or *gusA* cleave the uncolored substrates 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) or 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexyl ammonium salt (X-gluc), respectively, to produce products with blue color. Some advantages and disadvantages of these phenotypic markers have recently been discussed (Jansson 1995). For example, one useful application of *xylE* is the specific detection of intact or viable cells, because catechol 2,3-oxygenase is inactivated by oxygen and rapidly destroyed outside the cell (Prosser 1994).

Two disadvantages of the above mentioned marker genes are the potentially high background of marker enzyme activity in the indigenous microbial population and the requirement for growth and cultivation in the detection methods. DNA hybridization is another method that potentially could be used to detect these phenotypic marker genes as long as background levels are sufficiently low. Both *lacZ* and *gusA* have limited application in soil, however, because of their presence in the indigenous microbiota.

The *gfp* gene, encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is an attractive marker system with which to monitor bacterial cells

Table 4 The application of marker genes and methods used to detect introduced bacteria in bioremediation of xenobiotics

Marker gene	Microorganism	Detection method	References
lux or *lac*	*Pseudomonas cepacia* (2,4-D-degrader)	Non-selective plating, selective plating and autophotography	Masson et al. 1993
lux or *lac*	*Pseudomonas aeruginosa* (biosurfactant-producer)	Non-selective plating, selective plating, charge-coupled device (CCD)-enhanced detection, PCR and Southern blotting	Fleming et al. 1994b
lux	*P. aeruginosa* (biosurfactant-producer)	Bioluminescent-MPN (microplate assay), luminometry and CCD-enhanced detection	Fleming et al. 1994a
lux	*Alcaligenes eutrophus* strain H850 (PCB-degrader)	Selective plating and bioluminescence	van Dyke et al. 1996
lac	*Sphingomonas wittichii* strain RW1 (dibenzo-*p*-dioxin- and dibenzofuran-degrader)	Non-selective plating and selective plating	Megharaj et al. 1997
gfp or *lux*	*Pseudomonas* sp. strain UG14Gr (phenanthrene-degrader)	Non-selective plating, selective plating and CCD-enhanced detection	Errampalli et al. 1998
gfp	*Moraxella* sp. (*p*-nitrophenol-degrader)	Non-selective plating and selective plating	Tresse et al. 1998
xyl	*S. wittichii* strain RW1 (dibenzo-*p*-dioxin- and dibenzofuran-degrader)	Selective plating	Halden et al. 1999
gfp	*P. resinovorans* CA10 (carbazole- and dibenzo-*p*-dioxin-degrader)	Selective plating	Widada et al. 2002b
gfp or *luc*	*Arthobacter chlorophenolicus* A6 (4-chlorophenol-degrader)	Selective plating, luminometry, and flow cytometry	Elvang et al. 2001

in the environment. An advantage of the application of the *gfp* gene over that of other marker genes is the fact that the detection of fluorescence from GFP is independent of substrate or energy reserves (Tombolini et al. 1997). Since the *gfp* gene is eukaryotic in origin, it was first necessary to develop an optimized construct for expression of *gfp* in bacteria (Unge et al. 1999). Another reason that *gfp* is becoming so popular is that single cells tagged with *gfp* can easily be visualized by epifluorescence microscopy (Tombolini et al. 1997). In addition, fluorescent cells may be rapidly enumerated by flow cytometry (Ropp et al. 1995). The flow cytometer measures parameters related to size, shape and fluorescence of individual cells (Tombolini et al. 1997).

Another promising marker for determination of cellular metabolic activity is bacterial or eukaryotic luciferase. Bacterial luciferase catalyzes the following reaction: $RCHO + FMNH_2 + O_2 \rightarrow RCOOH + FMN + H_2O +$ light (490 nm), where R is a long chain aldehyde (e.g., *n*-decanal). Due to the requirement for reducing equivalent ($FMNH_2$), the bioluminescence output is directly related to the metabolic activity of the cells (Unge et al. 1999). The marker systems mentioned above for monitoring of augmented bacteria in bioremediation have been broadly applied (Table 4).

Recent development of methods increasing specificity of detection

A new approach that permits culture-independent identification of microorganisms responding to specified stimuli has been developed (Borneman 1999). This approach was illustrated by the examination of microorganisms that respond to various nutrient supplements added to environmental samples. A thymidine nucleotide analog, bromodeoxyuridine (BrdU), and specified stimuli were added to environmental samples and incubated for several days. DNA was then extracted from an environmental sample, and the newly synthesized DNA was isolated by immunocapture of the BrdU-labelled DNA. Comparison of the microbial community structures obtained from total environmental sample DNA and the BrdU-labelled fraction showed significantly different banding patterns between the nutrient supplement treatments, although traditional total DNA analysis revealed no notable differences (Borneman 1999). This approach provides a new strategy to permit identification of DNA from a stimulus- or substrate-responsive organism in environmental samples. Application of such an approach in bioremediation by using the desired xenobiotic as a substrate or stimulus added to an environmental sample may provide a robust strategy to discover novel catabolic genes involved in xenobiotic degradation.

It has been reported that bacteria belonging to the newly recognized phylogenetic groups are widely dis-

tributed in various environments (Dojka et al. 1998; Hugenholtz et al. 1998). The 16S rDNA sequences of these groups are very diverse and include mismatches to the bacterial universal primer designed from conserved regions in bacterial 16S rDNA sequences (Dojka et al. 1998; von Wintzingerode et al. 2000). Mismatches between PCR primer and a template greatly reduce the efficiency of amplification (von Wintzingerode et al. 1997). To overcome such problems, Watanabe et al. (2001) designed new universal primers by introducing inosine residues at positions where mismatches were frequently found. Using the improved primers, they could detect the phylotypes affiliated with *Verrucomicrobia* and candidate division OP11, which had not been detected by PCR-DGGE with conventional universal primers (Watanabe et al. 2001).

The number of bands in a DGGE gel does not always accurately reflect the number of corresponding species within the microbial community; one organism may produce more than one DGGE band because of multiple, heterogeneous rRNA operons (Cilia et al. 1996). Microbial community pattern analysis using 16S rRNA gene-based PCR-DGGE is significantly limited by this inherent heterogeneity (Dahllöf et al. 2000). As an alternative to 16S rRNA gene sequences in community analysis, Dahllöf et al. (2000) employed the gene for the β subunit of RNA polymerase (*rpoB*), which appears to exist in only one copy in bacteria. This approach proved more accurate compared with 16S rRNA gene-based PCR-DGGE for a mixture of bacteria isolated from red algae.

Recently, DNA microarrays have been developed and introduced for analyzing microbes and their activity in environmental samples (Cho and Tiedje 2002; Small et al. 2001; Wu et al. 2001). These are particularly powerful tools because of the large number of hybridizations that can be performed simultaneously on glass slides: over 100,000 spots per cm^2 can be accommodated (Kuipers et al. 1999). As with conventional dot blot hybridization, sample nucleic acids can be spotted onto the carrier material or reverse hybridization can be performed using immobilized probes. If PCR is involved, specific primers can be used to amplify partial or whole rRNA genes of the microorganisms of interest. Small et al. (2001) recently developed and validated a simple microarray method for the direct detection of intact 16S rRNA from unpurified soil extracts. In addition, it has been reported that DNA array technology is also a potential method for assessing the functional diversity and distribution of selected genes in the environment (Cho and Tiedje 2002; Wu et al. 2001).

The vast majority of environmental microorganisms have yet to be cultured. Consequently, a major proportion of the genetic diversity within nature resides in the uncultured organisms (Stokes et al. 2001). Isolation of these genes is limited by lack of sequence information, and PCR amplification techniques can be employed for the amplification of only partial genes. Thus a strategy to recover complete open reading frames from environmental DNA samples has been developed (Stokes et al. 2001). PCR assays targeted to the 59-base element family of recombination sites that flank gene cassettes associated with integrons were designed. Using such assays, diverse gene cassettes could be amplified from the vast majority of the environmental DNA samples tested. These gene cassettes contained a complete open reading frame, the majority of which were associated with ribosome binding sites. Such a strategy applied together with the BrdU strategy (Borneman 1999) should provide a robust method for discovering catabolic gene cassettes from environmental samples.

It is becoming increasingly apparent that the best solution for monitoring an introduced microorganism in the environment is to use either several markers simultaneously or multiple detection methods. Sometimes single markers or certain combinations of markers are not selective enough, such as *lacZY* used either alone or together with antibiotic selection. Even so, the use of antibiotic selection, in combination with bioluminescence, has been found to be very effective and useful for selection of low numbers of tagged cells (Jansson and Prosser 1997). A dual-marker system was developed for simultaneous quantification of bacteria and their activity by the *lux*AB and *gfp* gene products, respectively. Generally, the bioluminescence phenotype of the *lux*AB biomarker is dependent on cellular energy status. Since cellular metabolism requires energy, bioluminescence output is directly related to the metabolic activity of the cells. In contrast, the fluorescence of GFP has no energy requirement. Therefore, by combining these two biomarkers, total cell number and metabolic activity of a specific marked cell population could be monitored simultaneously (Unge et al. 1999).

In order to increase the specificity of detection, marker DNA may be detected in total DNA isolated and purified from an environmental sample by a variety of molecular-biology-based methods, such as gene probing, DNA hybridization, and quantitative PCR (Jansson 1995; Jansson and Prosser 1997).

Recently, we developed a rapid, sensitive, and accurate quantification method for the copy number of specific DNA in environmental samples by combining the fluorogenic probe assay, cPCR and co-extraction with internal standard cells (Widada et al. 2001). The internal standard DNA was modified by replacement of a 20-bp-long region responsible for binding a specific probe in fluorogenic PCR (TaqMan; Applied Biosystems, Foster City, Calif.). The resultant DNA fragment was similar to the corresponding region of the intact target gene in terms of G+C content. When used as a competitor in the PCR reaction, the internal standard DNA was distinguishable from the target gene by two specific fluorogenic probes with different fluorescence labels, and was automatically detected in a single tube using the ABI7700 sequence detection system (Applied Biosystems). By using an internal standard designed for cPCR, we found that the amplification efficiency of target and standard templates was quite similar and independent of the number of PCR cycles (Widada et al. 2001). The in-

ternal standard cell was used to minimize the variations in the efficiency of cell lysis and DNA extraction between the samples. A mini-transposon was used to introduce competitor DNA into the genome of a non-target bacterium in the same genus, and the resultant transformant was used as an internal standard cell. After adding a known amount of the internal standard cells to soil samples, we extracted the total DNA (co-extraction). Using this method, the copy number of the target gene in environmental samples can be quantified rapidly and accurately (Widada et al. 2001).

Biological containment strategies for bioaugmented microorganisms

Novel active biological containment (ABC) strategies have been developed to prevent the potentially undesirable spread of released microorganisms, particularly genetically engineered microorganisms (Molin et al. 1993; Molina et al. 1998; Ronchel et al. 1995, 1998, 2000). Several recent developments in molecular biology have led to the construction of new mechanisms to contain microorganisms once they have completed their intended jobs. One category of containment is known as a suicide system. For example, suicide systems have been developed for bacteria genetically modified to catabolize xenobiotics. In the absence of the chemical, a 'suicide gene' is induced and the introduced microorganism is eliminated from the population, although 100% elimination is never achieved. One such system places expression of the gene encoding streptavidin under the control of a cascade system that is ultimately controlled by the presence or absence of the growth substrate. In the presence of the growth substrate, expression of the streptavidin gene is repressed. In the absence of the growth substrate, streptavidin is produced, binds to D-biotin and kills the host organism (Szafranski et al. 1997).

Another ABC strategy is to reduce the spread of genes by the incorporation of a gene whose product kills non-immune recipients upon gene transfer (Fig. 2A). This system is based on the use of a killing gene, e.g., a porin-induced protein such as the one encoded by the *E. coli gef* gene, and a regulatory circuit that controls expression of the microorganisms that degrade a model pollutant – designed on the basis of the *P. putida* TOL plasmid meta-cleavage regulatory circuit. In this system, the *gef* gene was placed under control of the *lac* promoter. The repressor of the *lac* promoter, LacI, was placed under control of the meta-cleavage pathway promoter, Pm. In the presence of *m*-methylbenzoate, a *xylS* effector, expression of the *gef* gene was repressed. When the substrate disappeared, the *gef* gene was no longer repressed and the host cell was killed (Molin et al. 1993; Molina et al. 1998; Ronchel et al. 1995). However, this system had two main drawbacks: (1) the slow death of the bacterial cells in soil versus the fast killing rate in liquid cultures in laboratory assays, and (2) the appear-

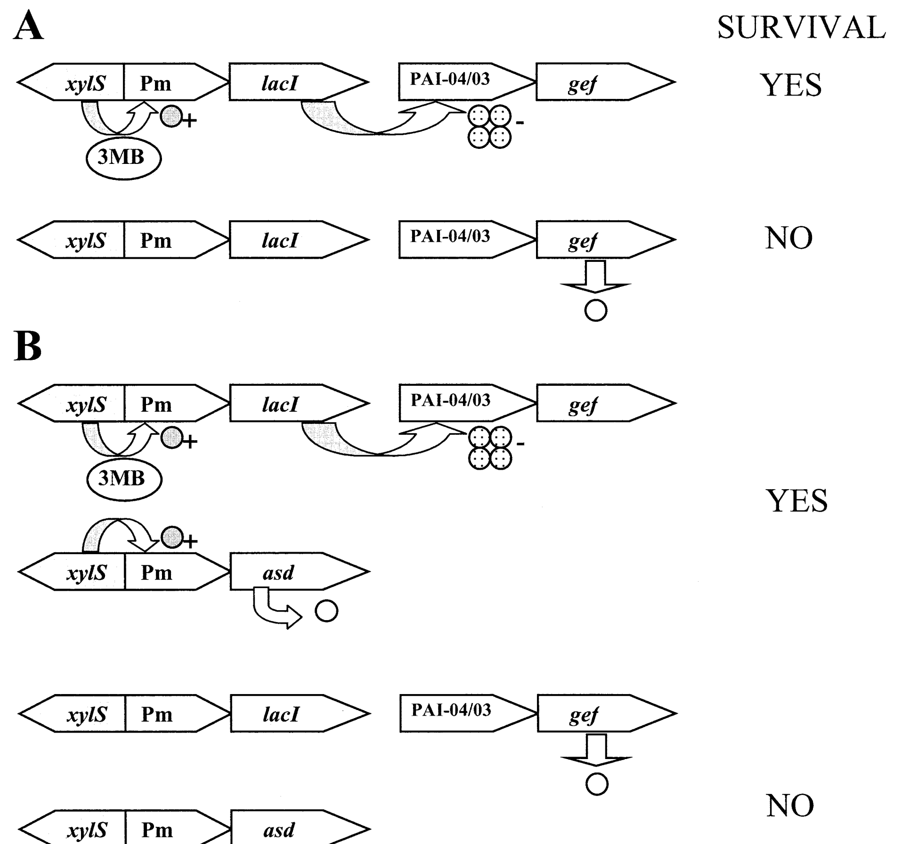

Fig. 2 Scheme of an active biological containment (ABC) model system (**A**) and the dual containment system (**B**) to control survival of bacteria by varying the availability of 3-methylbenzoate (3MB). *Pm* Promoter for the meta pathway, *lacI* repressor for the *lac* operon, *xylS* positive regulator of Pm, *asd* aspartate-β-semialdehyde dehydrogenase gene, $P_{A1\text{-}O4/O3}$ modified promoter for the *lac* operon, *gef* killing gene from *Escherichia coli* (Ronchel and Ramos 2001)

ance of mutants, at a rate of about 10^{-8} per cell and generation, that did not die after the pollutant had been exhausted (Ronchel and Ramos 2001). Therefore, Ronchel and Ramos (2001) reinforced the ABC system described above by including it in a Δ*asd P. putida* background (Fig. 2B). The *asd* gene product is involved in the biosynthesis of aspartate-β-semialdehyde, a key intermediate in the biosynthesis of diaminopimelic acid and of amino acids. A *P. putida* Δ*asd* mutant is viable only in complex medium supplemented with diaminopimelic acid, methionine, lysine, and threonine. They constructed a *P. putida* Δ*asd* strain, called MCR7, with a Pm::*asd* fusion in the host chromosome. This strain was viable in the presence of 3-methylbenzoate (3MB) because synthesis of the essential metabolites was achieved through XylS-dependent induction. An ABC system (Pm::*lacI*, *xylS*, Plac::*gef*) was incorporated into the host chromosome of the MCR7 strain to yield strain MCR8. The number of MCR8 mutants that escaped killing was below their detection limit ($<10^{-9}$ mutants per generation). The MCR8 strain survived and colonized rhizosphere soil with 3MB at a level similar to that of the wild-type strain. However, it disappeared in less than 20–25 days in soils without the pollutant, whereas an *asd*+, biologically contained counterpart such as *P. putida* CMC4 was still detectable in soils after 100 days (Ronchel and Ramos 2001).

Conclusions

The application of molecular-biology-based techniques in bioremediation is being increasingly used, and has provided useful information for improving of bioremediation strategies and assessing the impact of bioremediation treatments on ecosystems. Several recent developments in molecular techniques also provide rapid, sensitive, and accurate methods of analyzing bacteria and their catabolic genes in the environment. In addition, these molecular techniques have been used for designing active biological containment systems to prevent the potentially undesirable spread of released microorganisms, mainly genetically engineered microorganisms. However, a thorough understanding of the limitations of these techniques is essential to prevent researchers being led astray by their results.

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References

Allison DG, Ruiz B, San-Jose C, Jaspe A, Gilbert P (1998) Analysis of biofilm polymers of *Pseudomonas fluorescens* B52 attached to glass and stainless steel coupons. In: Abstracts of the General Meeting of the American Society for Microbiology, Atlanta, Georgia 98:325

Alvarez MJ, Depino AM, Podhajcer OL, Pitossi FJ (2000) Bias in estimations of DNA content by competitive polymerase chain reaction. Anal Biochem 28:87–94

Atlas M (1992) Molecular methods for environmental monitoring and containment of genetically engineered microorganisms. Biodegradation 3:137–146

Bakken LR, Lindahl V (1995) Recovery of bacterial cells from soil. In: Trevors JT, van Elsas JD (eds) Nucleic acids in the environment: methods and applications. Springer, Berlin Heidelberg New York, pp 9–27

Bastiaens L, Springael D, Dejonghe W, Wattiau P, Verachtert H, Diels L (2001) A transcriptional *luxAB* reporter fusion responding to fluorine in *Sphingomonas* sp LB126 and its initial characterizations for whole-cell bioreporter purposes. Res Microbiol 152:849–859

Bogardt AH, Hemmingsen BB (1992) Enumeration of phenanthrene-degrading bacteria by an overlayer technique and its use in evaluating petroleum-contaminated sites. Appl Environ Microbiol 58:2579–2582

Borneman J (1999) Culture-independent identification of microorganisms that respond to specified stimuli. Appl Environ Microbiol 65:3398–3400

Brockman FJ (1995) Nucleic-acid-based methods for monitoring the performance of in situ bioremediation. Mol Ecol 4:567–578

Cavalca L, Hartmann A, Rouard N, Soulas G (1999) Diversity of *tfd*C genes: distribution and polymorphism among 2,4-dichlorohenoxyacetic acid degrading soil bacteria. FEMS Microbiol Ecol 29:45–58

Chandler DP (1998) Redefining relativity: quantitative PCR at low template concentrations for industrial and environmental microbiology. J Ind Microbiol Biotechnol 21:128–140

Cho J-C, Tiedje JM (2002) Quantitative detection of microbial genes by using DNA microarrays. Appl Environ Microbiol 68:1425–1430

Cilia V, Lafay B, Christen R (1996) Sequence heterogeneities among 16S RNA sequences, and their effect on phylogenetic analyses at species level. Mol Biol Evol 13:415–461

Dahllöf I, Baillie H, Kjelleberg S (2000) *rpoB*-Based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. Appl Environ Microbiol 66:3376–3380

Dennis JJ, Zylstra GJ (1998) Plasposon: modular self-cloning mini-transposon derivatives for the rapid genetic analysis of Gram-negative bacterial genomes. Appl Environ Microbiol 64:2710–2715

Dijkmans R, Jagers A, Kreps S, Collard JM, Mergeay M (1993) Rapid method for purification of soil DNA for hybridization and PCR analysis. Microb Releases 2:29–34

Diviacco S, Norio P, Zentilin L, Menzo S, Clementi M, Biamonti G, Riva S, Falaschi A, Giacca M (1992) A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. Gene 122:313–320

Dojka MA, Hugenholtz P, Haack SK, Pace NR (1998) Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl Environ Microbiol 64:3869–3877

Dyke MI van, Lee H, Trevors JT (1996) Survival of *luxAB*-marked *Alcaligenes eutrophus* H850 in PCB-contaminated soil and sediment. J Chem Technol Biotechnol 65:115–122

Elsas JD van, Mantynen V, Wolters AC (1997) Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCP-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. Biol Fertil Soil 24:188–195

Elsas JD van, Rosado A, Moore AC, Karlson V (1998) Quantitative detection of *Sphingomonas chlorophenoliza* in soil via competitive polymerase chain reaction. J Appl Microbiol 85:463–471

Elvang AM, Westerberg K, Jernberg C, Jansson JK (2001) Use of green fluorescent protein and luciferase biomarkers to monitor survival and activity of *Arthrobacter chlorophenolicus* A6

cells during degradation of 4-chlorophenol in soil. Environ Microbiol 3:32–42

Ensley BD, Ratzkin BJ, Osslund TD, Simon MJ, Wackett LP, Gibson DT (1983) Expression of naphthalene oxidation genes in *Escherichia coli* results in biosynthesis of indigo. Science 222:167–169

Erb RW, Wagner-Doebler I (1993) Detection of polychlorinated biphenyl degradation genes in polluted sediments by directed DNA extraction and polymerase chain reaction. Appl Environ Microbiol 59:4065–4073

Errampalli D, Okamura H, Lee H, Trevors JT, van Elsas JT (1998) Green fluorescent protein as a marker to monitor survival of phenanthrene-mineralizing *Pseudomonas* sp. UG14Gr in creosote-contaminated soil. FEMS Microbiol Ecol 26:181–191

Fleming CA, Lee H, Trevors JT (1994a) Bioluminescent most-probable-number method to enumerate *lux*-marked *Pseudomonas aeruginosa* UG2Lr in soil. Appl Environ Microbiol 60:3458–3461

Fleming CA, Leung KT, Lee H, Trevors JT, Greer CW (1994b) Survival of *lux-lac*-marked biosurfactant-producing *Pseudomonas aeruginosa* UG2L in soil monitored by nonselective plating and PCR. Appl Environ Microbiol 60:1606–1613

Fleming JT, Sanseverino J, Sayler GS (1993) Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soil at town gas manufacturing sites. Environ Sci Technol 27:1068–1074

Fleming JT, Yao W-H, Sayler GS (1998) Optimization of differential display of prokaryotic mRNA: application to pure culture and soil microcosms. Appl Environ Microbiol 64:3698–3706

Fleming JT, Nagel AC, Rice J, Sayler GS (2001) Differential display of prokaryote messenger RNA and application to soil microbial communities. In: Rochelle PA (ed) Environmental molecular microbiology: protocol and applications. Horizon, Norfolk, England, pp 191–205

Foght JM, Westlake DWS (1996) Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. Biodegradation 7:353–366

Frostegard A, Courtois S, Ramisse V, Clerc S, Bernillon D, Le Gall F, Jeannin P, Nesme X, Simonet P (1999) Quantification of bias related to the extraction of DNA directly from soils. Appl Environ Microbiol 65:5409-5420

Fulthorpe RR, Wyndham RC (1989) Survival and activity of a 3-chlorobenzoate-catabolic genotype in a natural system. Appl Environ Microbiol 55:1584–1590

Goyal AK, Zylstra GJ (1996) Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from *Comamonas testosteroni* GZ39. Appl Environ Microbiol 62:230–236

Halden RU, Halden BG, Dwyer DF (1999) Removal of dibenzofuran, dibenzo-*p*-dioxin, and 2-chlorodibenzo-*p*-dioxin from soils inoculated with *Sphingomonas* sp. strain RW1. Appl Environ Microbiol 65:2246–2249

Hallier-Soulier S, Ducrocq V, Mazure N, Truffaut N (1996) Detection and quantification of degradative genes in soils contaminated by toluene. FEMS Microbiol Ecol 20:121–133

Hamann C, Hegemann J, Hildebrandt A (1999) Detection of polycyclic aromatic hydrocarbon degradation genes in different soil bacteria by polymerase chain reaction and DNA hybridization. FEMS Microbiol Lett 173:255–263

Hedlund BP, Geiselbrecht AD, Timothy JB, Staley JT (1999) Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas napthovorans*, sp. Nov. Appl Environ Microbiol 65:251–259

Herrick JB, Madsen EL, Batt CA, Ghiorse WC (1993) Polymerase chain reaction amplification of naphthalene-catabolic and 16S rRNA gene sequences from indigenous sediment bacteria. Appl Environ Microbiol 59:687–694

Holben WE, Schroeter BM, Calabrese VGM, Olsen RH, Kukor JK, Biederbeck UD, Smith AE, Tiedje JM (1992) Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. Appl Environ Microbiol 58:3941–3948

Hosein SG, Millette D, Butler BJ, Greer CW (1997) Catabolic gene probe analysis of an aquifer microbial community degrading creosote-related polycyclic aromatic and heterocyclic compounds. Microb Ecol 34:81–89

Hugenholtz P, Goebel BM, Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180:4765–4774

Hurt RA, Qiu X, Wu L, Roh Y, Palumbo AV, Tiedje JM, Zhou J (2001) Simultaneous recovery of RNA and DNA from soils and sediments. Appl Environ Microbiol 67:4495–4503

Jansson JK (1995) Tracking genetically engineered microorganisms in nature. Curr Opin Biotechnol 6:275–283

Jansson JK, Prosser JI (1997) Quantification of the presence and activity of specific microorganisms in nature. Mol Biotechnol 7:103–120

Khan AA, Wang R-F, Cao W-W, Doerge DR, Wennerstrom D, Cerniglia CE (2001) Molecular cloning, nucleotide sequence and expression of genes encoding a polycyclic aromatic ring dioxygenase from *Mycobacterium* sp. strain PYR-1. Appl Environ Microbiol 67:3577–3585

Kitagawa W, Suzuki A, Hoaki T, Masai E, Fukuda M (2001) Multiplicity of aromatic ring hydroxylation dioxygenase genes in a strong PCB degrader, *Rhodococcus* sp. strain RHA1 demonstrated by denaturing gel electrophoresis. Biosci Biotechnol Biochem 65:1907–1911

Knaebel DB, Crawford RL (1995) Extraction and purification of microbial DNA from petroleum-contaminated soils and detection of low numbers of toluene, octane and pesticide degraders by multiplex polymerase chain reaction and Southern analysis. Mol Ecol 4:579–591

Kuipers OP, de Jong A, Holsappel S, Bron S, Kok J, Hamoen LW (1999) DNA-microarrays and food-biotechnology. Antonie van Leeuwenhoek 76:353–355

Leser TD, Boye M, Hendriksen NB (1995) Survival and activity of *Pseudomonas* sp. strain B13(FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacterioplankton. Appl Environ Microbiol 61:1201–1207

Levesque MJ, La-Boissiere S, Thomas JC, Beaudet R, Villemur R (1997) Rapid method for detecting *Desulfitobacterium frappiri* strain PCP-1 in soil by the polymerase chain reaction. Appl Microbiol Biotechnol 47:719–725

Lindow SE (1995) The use of genes in the study of microbial ecology. Mol Ecol 4:555–566

Lloyd-Jones G, Laurie AD, Hunter DWF, Fraser R (1999) Analysis of catabolic genes for naphthalene and phenanthrene degradation in contaminated New Zealand soils. FEMS Microbiol Ecol 29:69–79

Madsen EL (1991) Determining in situ biodegradation: facts and challenges. Environ Sci Technol 25:1663–1673

Madsen EL, Sinclair JL, Ghiorse WC (1991) In situ biodegradation: microbiological patterns in a contaminated aquifer. Science 252:830–833

Marsh TL (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr Opin Microbiol 2:323–327

Masson L, Comeau Y, Brousseau R, Samson R, Greer C (1993) Construction and application of chromosomally integrated *lac-lux* gene markers to monitor the fate of a 2,4-dichlorophenoxyacetic acid-degrading bacterium in contaminated soils. Microb Releases 1:209–216

Meckenstock R, Steinle P, van der Meer JR, Snozzi M (1998) Quantification of bacterial mRNA involved in degradation of 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 from liquid culture and from river sediment by reverse transcriptase PCR (RT/PCR). FEMS Microbiol Lett 167:123–129

Megharaj M, Wittich R-M, Blasco R, Pieper DH, Timmis KN (1997) Superior survival and degradation of dibenzo-*p*-dioxin

and dibenzofuran in soil by soil-adapted *Sphingomonas* sp. strain RW1. Appl Microbiol Biotechnol 48:109–114

Meyer S, Moser R, Neef A, Stahl U, Kämpfer P (1999) Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. Microbiol 145:7131–1741

Miller DN, Bryant JE, Madsen EL, Ghiorse WC (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol 65:4715–4724

Molin S, Boe L, Jensen LB, Kristensen CS, Givskov M, Ramos JL, Bej AK (1993) Suicidal genetic elements and their use in biological containment of bacteria. Annu Rev Microbiol 47:139–166

Molina L, Ramos C, Ronchel MC, Molin S, Ramos JL (1998) Construction of an efficient biologically contained *Pseudomonas putida* strain and its survival in outdoor assays. Appl Environ Microbiol 64:2072–2078

Moller A, Jansson JK (1997) Quantification of genetically tagged cyanobacteria in Baltic Sea sediment by competitive PCR. Biotechniques 22:512–518

Moller A, Gustafsson K, Jansson JK (1994) Specific monitoring by PCR amplification and bioluminescence of firefly luciferase gene-tagged bacteria added to environmental samples. FEMS Microbiol Ecol 15:193–206

More MI, Herrick JB, Silva MC, Ghiorse WC, Madsen EL (1994) Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Appl Environ Microbiol 60:1572–1580

Moriarty F (1988) Exotoxicology: the study of pollutants in ecosystems, 2nd edn. Academic Press, New York

Moser R, Stahl U (2001) Insights into the genetic diversity of initial dioxygenases from PAH-degrading bacteria. Appl Microbiol Biotechnol 55:609–618

Muyzer G (1999) DGGE/TGGE: a method for identifying genes from natural ecosystems. Curr Opin Microbiol 2:317–322

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73:127–141

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nogales B, Moore ERB, Abraham W-R, Timmis KN (1999) Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. Environ Microbiol 1:199–212

Ogunseitan OA, Delgado IL, Tsai YL, Olson BH (1991) Effect of 2-hydroxybenzoate on the maintenance of naphthalene-degrading pseudomonads in seeded and unseeded soil. Appl Environ Microbiol 57:2873–2879

Okuta A, Ohnishi K, Harayama S (1998) PCR isolation of catechol 2,3-dioxygenase gene fragments from environmental samples and their assembly into functional genes. Gene 212:221–228

Picard C, Nesme X, Sinomet P (1996) Detection and enumeration of soil bacteria using the MPN-PCR technique. In: van Elsas JD (ed) Molecular ecology manual, vol 2. Kluwer, Dordrecht, pp 1–9

Prosser JI (1994) Molecular marker systems for detection of genetically engineered microorganisms in the environment. Microbiology 140:5–17

Ronchel MC, Ramos JL (2001) Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. Appl Environ Microbiol 67:2649–2656

Ronchel MC, Ramos C, Jensen LB, Molin S, Ramos JL (1995) Construction and behavior of biologically contained bacteria for environmental applications in bioremediation. Appl Environ Microbiol 61:2990–2994

Ronchel MC, Molina L, Witte A, Lutbiz W, Molin S, Ramos JL, Ramos C (1998) Characterization of cell lysis in *Pseudomonas putida* induced upon expression of heterologous killing genes. Appl Environ Microbiol 6:4904–4911

Ronchel MC, Ramos-Diaz MA, Ramos JL (2000) Retrotransfer of DNA in the rhizosphere. Environ Microbiol 2:319–323

Ropp JD, Donahue CJ, Wolfgang-Kimball D, Hooley JJ, Chin JYW, Hoffman RA, Cuthbertson RA, Bauer KD (1995) *Aequorea* green fluorescent protein and analysis by flow cytometry. Cytometry 21:309–317

Saano A, Tas E, Piippola S, Lindström K, van Elsas JD (1995) Extraction and analysis of microbial DNA from soil. In: Trevors JT, van Elsas JD (eds) Nucleic acids in the environment: methods and applications. Springer, Berlin Heidelberg New York, pp 49–67

Saito A, Iwabuchi T, Harayama S (2000) A novel phenanthrene dioxygenase from *Nocardiodes* sp. strain KP7: expression in *Escherichia coli*. J Bacteriol 182:2134–2141

Sato S, Nam J-W, Kasuga K, Nojiri H, Yamane H, Omori T (1997) Identification and characterization of the gene encoding carbazole 1,9a-dioxygenase in *Pseudomonas* sp. strain CA10. J Bacteriol 179:4850–4858

Sayler GS, Layton AC (1990) Environmental application of nucleic acid hybridization. Annu Rev Microbiol 44:625–648

Sayler GS, Shields MS, Tedford ET, Breen A, Hooper SW (1985) Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples. Appl Environ Microbiol 49:1295–1303

Schiwieger F, Tebbe CC (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl Environ Microbiol 64:4870–4876

Schneegurt-Mark A, Kulpa-Charler, F Jr (1998) The application of molecular techniques in environmental biotechnology for monitoring microbial systems. Biotechnol Appl Biochem 27:73–79

Selvaratnam S, Schoedel BA, McFarland BL, Kulpa CF (1995) Application of reverse transcriptase PCR for monitoring expression of the catabolic *dmpN* gene in a phenol-degrading sequencing batch reactor. Appl Environ Microbiol 61:3981–3985

Selvaratnam S, Schoedel BA, McFarland BL, Kulpa CF (1997) Application of the polymerase chain reaction (PCR) and reverse transcriptase/PCR for determining the fate of phenol-degrading *Pseudomonas putida* ATCC 11172 in a bioaugmented sequencing batch reactor. Appl Microbiol Biotechnol 47:236–240

Small J, Call DR, Brockman FJ, Straub TM, Chandler DP (2001) Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. Appl Environ Microbiol 67:4708–4716

Souza ML de, Wackett LP, Boundy-Mills KL, Mandelbaum RT, Sadowsky MJ (1995) Cloning, characterization, and expression of a gene region from *Pseudomonas* sp. strain ADP involved in the dechlorination of atrazine. Appl Environ Microbiol 61:3373–3378

Stokes HW, Holmes AJ, Nield BS, Holley MP, Nevalainen KMH, Mabbutt BC, Gillings MR (2001) Gene cassette PCR: sequence-independent recovery of entire genes from environmental DNA. Appl Environ Microbiol 67:5240–5246

Szafranski P, Mello CM, Sano T, Smith CL, Kaplan DL, Cantor CR (1997) A new approach for containment of microorganisms: dual control of streptavidin expression by antisense RNA and the T7 transcription system. Proc Natl Acad Sci USA 94:1059–1063

Taliani MR, Roberts SC, Dukek BA, Pruthi RK, Nichols WL, Heit JA (2001) Sensitivity and specificity of denaturing high-pressure liquid chromatography for unknown protein C gene mutations. Genet Test 5:39–44

Taranenko NI, Hurt R, Zhou J, Isola NR, Huang H, Lee SH, Chen CH (2002) Laser desorption mass spectrometry for microbial DNA analysis. J Microbiol Methods 48:101–106

Tchelet R, Meckenstock R, Steinle P, van der Meer JR (1999) Population dynamics of an introduced bacterium degrading chlorinated benzenes in a soil column and in sewage sludge. Biodegradation 10:113–125

Tombolini R, Unge A, Davey ME, de Bruijn FJ, Jansson JK (1997) Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. FEMS Microbiol Ecol 22:17–28

Torsvik V, Daae FL, Goksoyr J (1995) Extraction, purification and analysis of DNA from soil bacterial. In: Trevors JT, van Elsas JD (eds) Nucleic acids in the environment: methods and applications. Springer, Berlin Heidelberg New York, pp 29–48

Tresse O, Errampalli D, Kostrzynska M, Leung KT, Lee H, Trevors JT, van Elsas JD (1998) Green fluorescent protein as a visual marker in a *p*-nitrophenol degrading *Moraxella* sp. FEMS Microbiol Lett 164:187–193

Trevors JT, van Elsas JD (1995) Introduction to nucleic acids in the environment: methods and applications. In: Trevors JT, van Elsas JD (eds) Nucleic acids in the environment: methods and applications. Springer, Berlin Heidelberg New York, pp 1–7

Unge A, Tombolini R, Mølbak L, Jansson JK (1999) Simultaneous monitoring of cell number and metabolic activity of specific bacterial populations with a dual *gfp-luxAB* marker system. Appl Environ Microbiol 65:813–821

Walia S, Khan A, Rosenthal N (1990) Construction and applications of DNA probes for detection of polychlorinated biphenyl-degrading genotypes in toxic organic-contaminated soil environments. Appl Environ Microbiol 56:254–259

Watanabe K, Teramoto M, Futamata H, Harayama S (1998) Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. Appl Environ Microbiol 64:4396–4402

Watanabe K, Kodama Y, Harayama S (2001) Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. J Microbiol Methods 44:253–262

Watts JEM, Wu Q, Schreier SB, May HD, Sowers KR (2001) Comparative analysis of polychlorinated biphenyl-dechlorinating communities in enrichment cultures using three different molecular screening techniques. Environ Microbiol 3:710–719

Weller R, Ward DM (1989) Selective recovery of 16S ribosomal RNA sequences from natural microbial communities in the form of complementary DNA. Appl Environ Microbiol 55:1818–1822

Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H, Omori T (2001) Quantification of carbazole 1,9a-dioxygenase gene by real-time competitive PCR combined with co-extraction of internal standards. FEMS Microbiol Lett 202:51–57

Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H, Omori T (2002a) Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. Appl Microbiol Biotechnol 58:202–209

Widada J, Nojiri H, Yoshida T, Habe H, Omori T (2002b) Enhanced degradation of carbazole and 2,3-dichlorodibenzo-*p*-dioxinin soils by *Pseudomonas resinovorans* strain CA10. Chemosphere (in press)

Wilson MS, Bakerman C, Madsen EL (1999) In situ, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. Appl Environ Microbiol 65:80–87

Wintzingerode F von, Gobel UB, Stackkenbrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213–229

Wintzingerode F von, Landt O, Ehrlich A, Gobel UB (2000) Peptide nucleic acid-mediated PCR clamping as a useful supplement in the determination of microbial diversity. Appl Environ Microbiol 66:549–557

Wu L, Thompson DK, Li G, Hurt RA, Tiedje JM, Zhou J (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl Environ Microbiol 67:5780–5790

Yakimov MM, Giuliano L, Timmis KN, Golyshin PN (2001) Upstream-independent ribosomal RNA amplification analysis (URA): a new approach to characterizing the diversity of natural microbial communities. Environ Microbiol 3:662–666

Yeates C, Holmes AJ, Gillings MR (2000) Novel forms of ring-hydroxylating dioxygenases are widespread in pristine and contaminated soils. Environ Microbiol 2:644–653