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MINI-REVIEW

M. Dua · A. Singh · N. Sethunathan · A. K. Johri

Biotechnology and bioremediation: successes and limitations

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Abstract With advances in biotechnology, bioremediation has become one of the most rapidly developing fields of environmental restoration, utilizing microorganisms to reduce the concentration and toxicity of various chemical pollutants, such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phthalate esters, nitroaromatic compounds, industrial solvents, pesticides and metals. A number of bioremediation strategies have been developed to treat contaminated wastes and sites. Selecting the most appropriate strategy to treat a specific site can be guided by considering three basic principles: the amenability of the pollutant to biological transformation to less toxic products (biochemistry), the accessibility of the contaminant to microorganisms (bioavailability) and the opportunity for optimization of biological activity (bioactivity). Recent advances in the molecular genetics of biodegradation and studies on enzyme-tailoring and DNA-shuffling are discussed in this paper.

Introduction

The ever-growing list of chemical contaminants released into the environment on a large scale includes numerous aliphatic and aromatic compounds, such as petroleum hydrocarbons, halogenated and nitroaromatic compounds and phthalate esters. These compounds enter the environment through many different paths. As components of fertilizers, pesticides and herbicides, some are distributed by direct application. Combustion processes release others, such as polycyclic aromatic hydrocarbons (PAHs), dibenzo-*p*-dioxins and dibenzofurans. The local concentration of a contaminant depends on the amount present and the rate at which the compound is released, its stability in the environment under both aerobic and anaerobic conditions, the extent of its dilution in the environment, the mobility of the compound in a particular environment and its rate of biological or non-biological degradation (Harayama 1997; Ellis 2000; Janssen et al. 2001).

The term “bioremediation” has been used to describe the process of using microorganisms to degrade or remove hazardous components of the wastes from the environment (Glazer and Nikaido 1995). Biodegradation and its application in bioremediation of organic pollutants have benefited from the biochemical and molecular studies of microbial processes (Lal et al. 1986; Fewson 1988; Bollag and Bollag 1992; Johri et al. 1996). Various authors have reviewed the subject of biodegradation of organopollutants over the past decade (Sangodkar et al. 1989; Chaudhary and Chapalamadugu 1991; Van der Meer et al. 1992; Deo et al. 1994; Kumar et al. 1996; Johri et al. 1999; Janssen et al. 2001). Biotransformation of organic contaminants in the natural environment has been extensively studied to understand microbial ecology, physiology and evolution for their potential in bioremediation (Bouwer and Zehnder 1993; Chen et al. 1999; Johan et al. 2001; Mishra et al. 2001; Watanabe 2001). Here, recent advances in the molecular aspects of biodegradation and studies on enzyme-tailoring and DNA-shuffling are discussed.

Chemical pollution: a grim face

A significant amount of a wide variety of industrial organic chemicals are released into the environment delib-

M. Dua · A.K. Johri (✉)
Department of Zoology, University of Delhi, Delhi 110007, India
e-mail: ajohri@rics.bwh.harvard.edu
Tel.: +1-617-5250359, Fax: +1-617-7311541

A. Singh
Department of Biology, University of Waterloo, Waterloo, N2L 3G1, Ontario, Canada

N. Sethunathan
CSIRO Land and Water, PMB 2, Glen Osmond, SA 5064, Australia

Present addresses:
A.K. Johri, Channing Laboratory, Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115, USA

M. Dua, Department of Biomedical Research, Division of Hematology/Oncology, St. Elizabeth Medical Center, School of Medicine, Tufts University, Boston, MA 02135, USA

erately, to function as pesticides or to preserve wood or insulate electric transformers. Others are released accidentally or disposed of as waste. Many of these chemicals, produced on a large scale as part of the normal activities of industrialized societies, are considered hazardous to humans, plants and animals. The persistence of industrial chemicals in soil and water varies widely. Those that can be degraded by microorganisms or decompose spontaneously under natural conditions may disappear in weeks, while others may persist for years (Gibson and Parales 2000). Many pesticides once used in massive amounts have long half-lives in the soil, e.g. the environmental persistence of the insecticide DDT ranges over 3–10 years, that of chlordane 2–4 years and HCH persists for up to 11 years (Lichtenstein and Polivka 1957). Two fundamental questions are being asked – how to dispose of the large quantities of waste that are continually being produced; and how to remove the toxic compounds that have been accumulating at dump sites, in the soil and in water systems?

Although regulations are strictly enforced in developed countries like the United States and most of the European countries to meet the challenges of environmental contamination, these regulations often remain unenforced in most of the developing countries. Many hazardous waste dumpsites are unlined, with no barrier between the waste and ground water. These dumpsites may contain solid or liquid waste or both; and either a single compound may be present, or there may be a mixture of closely related compounds, or an unknown combination of unrelated substances. Cleaning up such sites is often not only technically challenging but also very expensive. Considerable pressure encourages the adoption of waste management alternatives to burial, the traditional means of disposing of solid and liquid wastes. Approaches such as air-stripping (to remove volatile compounds) and incineration have been used. However, where one or more compounds contaminate a large area in low but significant concentrations, such methods are either very costly or simply not feasible. Currently, researchers are testing a number of technological strategies – including biotechnological schemes – to deal with large-scale waste sites (Blackburn and Hafker 1993; Singh et al. 2001). In such schemes, microorganisms can provide an effective alternative.

Microorganisms: an asset!

Microorganisms excel at using organic substances, natural or synthetic, as sources of nutrients and energy. The explanation for their remarkable range of degradative abilities is that, by the time human beings came on the scene, microorganisms had already coexisted for billions of years with an immense variety of organic compounds. The vast diversity of potential substrates for growth led to the evolution of enzymes capable of transforming many unrelated natural organic compounds by many different catalytic mechanisms. The resulting giant "library" of microbial enzymes serves as raw material for further evolution whenever a new chemical becomes available (Butler and Mason 1997; Ellis 2000).

Depending on their behavior in the environment, organic compounds are often classified as biodegradable, persistent or recalcitrant. A biodegradable organic compound is one that undergoes a biological transformation (Blackburn and Hafker 1993; Liu and Suflita 1993). A persistent organic compound does not undergo biodegradation in certain environments; and a recalcitrant compound resists biodegradation in a wide variety of environments. While partial biodegradation is usually an alteration by a single reaction, primary biodegradation involves a more extensive chemical change. Mineralization is a parallel term to biodegradation, referring to complete degradation to the end products of CO_2, water and other inorganic compounds.

Microorganisms with the ability to degrade a wide variety of compounds, like benzene, phenol, naphthalene, atrazine, nitroaromatics, biphenyls, polychlorinated biphenyls (PCBs) and chlorobenzoates, have been isolated and characterized (Sangodkar et al. 1989; Dickel et al. 1993; Faison 2001). Although simple aromatic compounds are biodegradable by a variety of degradative pathways, their halogenated counterparts are more resistant to bacterial attacks and often necessitate the evolution of novel pathways (Chakrabarty 1982; Engasser et al. 1990). Many of the environmentally important chemicals introduced for industrial uses are halogenated and halogenation, which is often implicated as a reason for persistence. Among the halogenated compounds, the chlorinated compounds are the most extensively studied (Chaudhary and Chapalamadugu 1991; Cork and Krueger et al. 1992). The presence of chlorine atoms on the aromatic nucleus is known to greatly retard the rate of degradation.

Most of the information available on the biodegradation of chlorinated compounds is on oxidative degradation, since aerobic culture techniques are relatively simple, compared with anaerobic culture methods. Also, aerobic processes are considered the most efficient and generally applicable (Adriaens and Vogel 1995). Aerobic degradation is dependent on the presence of molecular oxygen and is catalyzed by enzymes that have evolved for the catabolism of natural substrates and exhibit low specificities. The oxidation of the molecule takes place without the removal of the chlorine atom in the first step. Depending upon the type of enzyme catalyzing the reaction, either one (monooxygenase) or two (dioxygenase) oxygen atoms are inserted into the molecule via an electrophilic attack on an unsubstituted carbon atom. Anaerobic degradation proceeds via reductive dehalogenation, wherein an electron transfer to the halogenated compound results in the replacement of a halogen with a hydrogen atom (Janssen et al. 2001).

Biotechnology: any help?

Biotechnological processes for the bioremediation of chemical pollutants offer the possibility of in situ treat-

ments and are mostly based on the natural activities of microorganisms. Biotechnological processes to destroy hazardous wastes offer many advantages over physicochemical processes. When successfully operated, biotechnological processes may achieve complete destruction of organic wastes. However, an important factor limiting the bioremediation of sites contaminated with certain hazardous compounds is the slow rate of degradation (Iwamoto and Nasu 2001). This slow degradation rate often limits the practicality of using microorganisms in remediating contaminated sites. This is an area where genetic engineering can make a marked improvement. Molecular techniques can be used to increase the level of a particular protein or enzyme or series of enzymes in bacteria with an increase in the reaction rate (Chakrabarty 1986). The easiest way to create an appropriate genetically engineered strain is to begin with an organism that already possesses much of the necessary degradative enzymatic machinery.

Genetically engineered microorganisms

Advances in genetic and protein engineering techniques have opened up new avenues to move towards the goal of genetically engineered microorganisms (GEMs) to function as "designer biocatalysts", in which certain desirable biodegradation pathways or enzymes from different organisms are brought together in a single host with the aim of performing specific reactions. A number of opportunities for improving degradation performance using GEMs have been described (Timmis and Piper 1999). The enzyme 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus* RDC1 (*etbC*-encoded) grown on ethylbenzene contains the broadest substrate specificity of any *meta*-cleavage dioxygenase identified in *Rhodococcus* strains (Masai et al. 1995). The co-expression of two 2,3-dihydroxybiphenyl 1,2-dioxygenases encoded by *bphC* and *etbC* genes in *Rhodococcus* strain RHA1 grown either on biphenyl or ethylbenzene has been found to increase the spectrum of PCB substrates (Hauschild et al. 1996).

A gene encoding *s*-triazine hydrolase (for dechlorination and deamination of de-ethylatrazine and de-isopropylatrazine) was transferred from *R. corallinus* to *Rhodococcus* TE1 (Chen and Mulchandani 1998). This resulted in the complete degradation of atrazine to cyanuric acid by the recombinant *Rhodococcus*. Recombinant *Escherichia coli* harboring and expressing the phenol hydroxylase gene from *Pseudomonas putida* BH could efficiently degrade trichloroethylene (TCE; Fujita et al. 1995). A *P. fluorescens* strain expressing the toluene *o*-monooxygenase genes from *Burkholderia cepacia* PR1 could very efficiently degrade TCE from contaminated soil (Yee et al. 1998). The genes for phenol hydroxylase and catechol 2,3-dioxygenase activities were also cloned and expressed independently and constitutively under various conditions in *Alcaligenes eutrophus* JMP134 (Hauschild et al. 1996). The expression of the gene(s) encoding phenol hydroxylase activity was found to affect the oxidative removal of TCE. Cloning of the *tod* gene encoding toluene dioxygenase into *Deinococcus radiodurans* enabled the bacterium to oxidize toluene, chlorobenzene, 3,4-dichloro-1-butene and indole. The engineered *D. radiodurans* was successfully applied to the bioremediation of mixed wastes containing both radionuclides and organic solvents (Lange et al. 1998).

Erb and Wagner-Dobber (1993) demonstrated the enhanced degradation of a mixture of phenols, using a genetically engineered pseudomonad in a laboratory-scale sewage plant fed with a mixture of chlorophenols and 4-methylphenol. They also showed that the microbiota can be protected from the lethal effects of the pollutants and thereby assure maintenance of the waste-treatment process. Researchers from the University of Tennessee, in collaboration with Oak Ridge National Laboratory, recently conducted bioremediation field tests, using a genetically modified strain of *P. fluorescens* strain HK44 (Sayler et al. 1999; Ripp et al. 2000). Strain HK44 was designed for the purpose of sensing an environmental contaminant and responding to it through bioluminescence signaling, to be used as an online tool for in situ monitoring of the bioremediation process (Sayler and Ripp 2000).

Enzyme-tailoring and DNA-shuffling

Through the genetic engineering of metabolic pathways, it is possible to extend the range of substrates that an organism can utilize. Aromatic hydrocarbon dioxygenases, belonging to a large family of Rieske non-heme iron oxygenases, have a broad substrate specificity and catalyze enantiospecific reactions with a wide range of substrates. These characteristics make these enzymes attractive synthons for the production of industrially and medically important chiral chemicals and provide essential information for the development of bioremediation technology (Gibson and Parales 2000). Aromatic hydrocarbon dioxygenases belong to a large family known as aromatic-ring hydroxylating dioxygenases (Butler and Mason 1997). All members of this family have one or two electron transport proteins preceding their oxygenase components. The family also contains monooxygenases and other enzymes that do not hydroxylate aromatic rings. The crystal structure of naphthalene dioxygenase has confirmed the long-suspected presence of a Rieske (2Fe–2S) cluster and mononuclear iron in each alpha subunit. On the basis of this study, changing the family name to Rieske non-heme iron oxygenases has been suggested (Gibson and Parales 2000), reserving the designations dioxygenases and monooxygenases for systems that are unambiguous.

Hybrid dioxygenases that have acquired enhanced degradation capabilities for PCBs, TCE and some other aromatic hydrocarbons were recently reviewed (Furukawa 2000). *P. putida* carrying the TOL plasmid could grow on a variety of alkylbenzoates, except 4-ethyl-

benzoate. Lehrbach et al. (1984) successfully cloned catabolic genes *xylD* and *xylL* from the TOL plasmid pWW0-161, coding for toluate-1,2-dioxygenase and dihydroxybenzoic acid dehydrogenase, and *nahG* from the NAH plasmid NAH7, coding salicylate hydroxylase, to construct *Pseudomonas* sp. B13 derivatives capable of utilizing 4-chlorobenzoate, 3,5-dichlorobenzoate, salicylate and chlorosalicylate as new growth substrates. Site-directed mutagenesis of the *Pseudomonas* sp. strain LB400 *bphA* gene resulted in an enzyme combining the broad congeners-specificity of LB400 with increased activity against several congeners characteristic of *P. pseudoalcaligenes* KF707 (Erickson and Mondello 1993). Biphenyl dioxygenases from *B. cepacia* LB400 and *P. pseudoalcaligenes* KF707 are structurally very similar, but exhibit different specificity for PCBs (Mondello et al. 1997). A variety of chimeric *bphA1* genes were constructed by exchanging four common restriction fragments between the KF707 *bphA1* and the LB400 *bphA1* genes to obtain enzyme variants with the capability to hydroxylate double *ortho*- and double *para*-substituted PCBs, thus combining the substrate range of both parental enzymes (Kimura et al. 1997).

The cloning of the transposable chlorobenzoate 3,4-dioxygenase genes of *Alcaligenes* sp. strain BR60, into *E. coli* gave rise to two proteins (Nakatsu and Wyndham 1993). Chlorobenzenes are generally not easily metabolized in the natural environment. It was found that the bacteria using chlorobenzenes as their sole source of carbon and energy had undergone a process of recombining or assembly of existing genetic material, called “horizontal expansion” (Van der Meer 1997). This horizontal expansion resulted in altered gene functions to accommodate chlorobenzenes as their substrates. The possibilities for low-frequency horizontal transfer of the self-transmissible chlorocatechol degradative genes (*clc*) from *Pseudomonas* sp. strain B13 were investigated in activated sludge microcosms (Ravatn et al. 1998). When the *clc* genes were transferred into an appropriate recipient bacterium such as *P. putida* F1, a new metabolic pathway for chlorobenzene degradation was formed. The substrate range of toluene/*o*-xylene monooxygenase (ToMO) was extended to include even the aliphatic chlorinated compounds. *E. coli* JM109 expressing the ToMO genes could degrade TCE, 1,1-dichloroethylene and chloroform, in addition to toluene and xylene (Chauhan et al. 1998). The genetic investigations of *Ralstonia* sp. JS705 revealed a unique combination of genes for chlorocatechol degradation and genes for a benzene–toluene type of aromatic ring dioxygenase. It was suggested that horizontal gene transfer and genetic recombination of the existing genes were the mechanisms that led to the evolution of the chlorobenzene catabolic pathway (Van der Meer et al. 1998).

The role of catabolic plasmids and the transposons harboring catabolic genes has been reviewed (Clarke 1980; Wyndham et al. 1994; Tan 1999). Many catabolic genes have been found associated with insertion sequences, suggesting that these clusters could be rapidly disseminated among the bacterial population to expand the substrate range and to evolve novel degradative pathways. Catabolic enzymes can be engineered for the enhancement of degradation rates or to broaden substrate specificity (Chen et al. 1999; Timmis and Piper 1999). Site-directed mutagenesis or gene-shuffling guided by computer-assisted modeling of three-dimensional protein structures facilitates the tailoring of enzymatic properties (Singh 1999). Haloalkane dehalogenase is the initial enzyme responsible for the substitution of a terminal chlorine atom by a hydroxyl group. The catalytic mechanism of this reaction is well understood and the enzyme has been shown to dechlorinate a wide range of substrates (Verschueren et al. 1993; Stevenson et al. 1998). Using structural information available for the enzyme–substrate, enzyme–intermediate and enzyme–product complexes as a guide, the bulky amino acids lining the catalytic cavity were replaced with alanine (increasing active-site volume; Verschueren et al. 1993). The resulting variants were several-fold more active in dechlorinating dichlorohexane. However, no mutant tested could utilize more bulky substrates, such as TCE (Holloway et al. 1998).

It has been suggested that sequence diversity in catabolic genes for the degradation of aromatic compounds is rather broad; and new genes and genetic organizations are still being discovered (Harford-Cross et al. 2000; Pieper and Reineke 2000). The construction of hybrid nitrotoluene dioxygenases was helpful in identifying the carboxy-terminal region of the large subunit of this enzyme as being critical for substrate specificity (Parales et al. 1998). The crystal structure of naphthalene dioxygenase was found helpful for the engineering of aromatic dioxygenases by rational design (Kauppi et al. 1998). Saito et al. (2000) recently observed that *Nocardioides* grew on phenanthrene but not on naphthalene. The genetic organization of the catabolic genes that encodes phenanthrene dioxygenase belongs to the group of aromatic ring-hydroxylating dioxygenases. Site-directed mutagenesis studies with naphthalene dioxygenase showed the importance of Phe352 in determining the regioselectivity of the enzyme with naphthalene, biphenyl and phenanthrene as substrates (Parales et al. 2000). Structural studies exhibited the presence of an indole–oxygen adduct at the active site of naphthalene dioxygenase (Carredano et al. 2000).

However, rational design approaches can fail due to unexpected influences exerted by the substitution of one or more amino acid residues. Rational (site-directed) approaches are also restrictive, because they allow the exploration of only a very limited sequence space at a time. Some other methods, such as DNA-shuffling, random priming or staggered extension processes can therefore be the ideal choices to direct the evolution of enzymes or pathways with highly specialized traits (Stemmer 1994; Kuchner and Arnold 1997; Harayama 1998; Shao et al. 1998; Zhao et al. 1998). Irrational approaches do not require prior extensive structural or biochemical data. When combined with focused selection or screening, ir-

rational approaches offer useful alternatives for generating both the desired improvements and a database for future rational approaches to protein design. Perhaps the most powerful and promising utility of DNA-shuffling is in the crossbreeding of genes between diverse classes of species, because of the extended sequence space that can be explored (Crameri et al. 1998).

By random shuffling of DNA segments between the large subunit of two wild-type biphenyl dioxygenases, variants were obtained with extended substrate range of biphenyl dioxygenases toward PCBs (Kumamaru et al. 1998; Bruhlmann and Chen 1999). Several variants had extended substrate ranges for PCBs exceeding those of the two parental enzymes. Irrational strategies have also been employed to amplify homologous biodegradative enzymes and incorporate them into recombinant enzymes without characterization of the host microorganisms. This approach was recently demonstrated by the modification of catechol 2,3-dioxygenase (Okuta et al. 1998). Degenerate primers were used to amplify the central segment of the enzyme present in a consortium of microorganisms derived from soil and seawater samples. A second round of PCR incorporated the amplified central domains into the 5′ and 3′ arms of the *nahH* gene.

Construction of bacteria with multiple pathways

Genetic engineering also permits the combination of several degradative activities within a single host organism. If a single strain is constructed to perform several related or unrelated metabolic activities, the efficiency and predictability of the process may be significantly enhanced. Such recombinant strains may be useful for the bioremediation of recalcitrant compounds (Brenner et al. 1994). Requirements for the design of bacteria with multiple pathways for use in bioremediation have been described in recent articles (Lau and Lorenzo 1999; Gibson and Parales 2000).

The catabolic pathway for the total degradation of PCBs is encoded by two different sets of genes that are not normally found in the same organism. As the catecholic intermediates of both pathways are frequently inhibitory to each other, incompatibilities result. Therefore, hybrid strains have been constructed that can metabolize simple chlorobiphenyls through complementary pathways by comprising the biphenyl, benzoate and chlorocatechol genes of the parental strains. By creating recombinant strains between *P. pseudoalcaligenes* KF707 and *B. cepacia* LB400 *bph* genes, not only was an enhanced degradation of PCBs demonstrated, but also the simultaneous degradation was shown of single aromatic hydrocarbons, such as benzene and toluene, which are otherwise poor substrates for the original biphenyl dioxygenases (Kumamaru et al. 1998). *P. aeruginosa* 142, isolated from a PCB-degrading consortium, was shown to be able to grow on chlorobenzoates (Romanov and Hausinger 1994). However, the presence of a broad substrate specificity and high-regiospecificity oxidative dehalogenation pathway involving an *ortho*-halobenzoate 1,2-dioxygenase provided the bacterium with the ability to utilize mixtures of chlorobenzoates and PCBs.

After inoculating *P. aeruginosa* JB2, a chlorobenzoate degrader, into soil having indigenous biphenyl degraders, two recombinant strains, *P. aeruginosa* JB2-3 and JB2-M, were isolated after some time (Foght et al. 1996). These strains were able to degrade both biphenyls and chlorobenzoates. Genetic analysis indicated that the *bph* genes were transferred to the original *P. aeruginosa* JB2 from the indigenous biphenyl degraders. The construction of hybrid *Pseudomonas* strains, JR1A::ipb and CBS-3::ipb, was achieved by inserting an *ibp* module (for degradation of isopropylbenzene) into the genomes of different *Pseudomonas* strains. The hybrid strains could effectively degrade TCE along with isopropylbenzene (Berendes et al. 1998).

Recently, Timmis and Piper (1999) suggested a strategy for designing organisms with novel pathways and the creation of a bank of genetic modules encoding broad-specificity enzymes or pathway segments that can be combined at will to generate new or improved activities. The use of appropriate regulatory circuits can enhance substrate flux through these designed pathways; and rationally engineering the pathway branch-points can avoid or reduce substrate misrouting. Using genes encoding the biosynthetic pathway of biosurfactants can enhance biodegradation rates by improving the bioavailability of the substrates; and genes encoding resistance to critical stress factors may enhance both the survival and the performance of designed catalysts.

Development of probes and biosensors

Methods for the rapid and specific identification of microorganisms within their natural environments are being developed. Classic methods are time-consuming and only work for a limited number of microorganisms; and the use of fluorescence in situ hybridization for the identification of microorganisms has been reviewed (Amann et al. 2001). An increasing need to develop new methods for characterization of microorganisms able to degrade pesticides and other xenobiotics has led to the use of molecular probes to identify, enumerate and isolate microorganisms with degradative potential.

Several DNA probes for PCB-degrading genotypes have been constructed from PCB-degrading bacteria. The usefulness of DNA probes was demonstrated in detecting specific PCB-degrading bacteria, abundance of PCB degrading genotypes and genotypic diversity among PCB-degrading bacteria in toxic, chemical-polluted environments (Walia et al. 1990). In a more specific study, the *bphC* gene was detected without prior cultivation of microorganisms by extraction of total DNA, PCR amplification of *bphC* sequences and detection with specific gene probes (Erb and Wagner-Dobber 1993). This method proved to be very sensitive, as the *bphC* gene of *P. paucimobilis* UT26 and *Pseudomonas* LB400

could be detected in as few as 100 cells/g sediment. It was the first report on direct amplification and detection of a chromosomally encoded, single copy gene from a highly specialized subpopulation of the total microbial community in natural sediments. A simple means to develop strain-specific DNA probes was developed by amplification of genomic DNA via repetitive sequence-based PCR, using primers specific for repetitive extragenic palindromic elements, followed by cloning of the amplified fragments (Matheson et al. 1997). The cloned fragments were screened to identify those which were strain-specific; and these were used as probes for total genomic DNA isolated from microbial communities. One such strain-specific probe for *B. cepacia* G4 was developed and used for microbial studies during TCE removal in an aquifer sediment microcosm (Matheson et al. 1997).

Recently, Fode-Vaughan et al. (2001) showed the detection of bacteria in environmental samples by direct PCR without DNA extraction. It has been further suggested that PCR provides a rapid, simple and sensitive method for detecting and quantifying bacteria in environmental samples. The use of fluorescence in situ hybridization (FISH) in wastewater treatment has been reported (Whiteley and Bailey 2000). For example, an industrial bioremediation system designed for the removal of phenolic compounds was shown by FISH to be dominated by members of the *Cytophaga*, *Flavobacterium* cluster and proteobacteria. Of these two groups, only the latter was positively correlated with phenol degradation. FISH was recently applied to the study of microbial communities in acid mine environments, in the context of the prevailing geochemical and mineralogical conditions (Bond et al. 2000). The technique facilitated the in situ identification of a new species of acidophilic archaean that is an important contributor to acid mine drainage (Edwards et al. 2000). Radajewski et al. (2000), using molecular phylogenetic analyses of isotope-labeled DNA probes, successfully identified two novel methanotrophs that actively degrade methane under environmental conditions.

Newly developed bioluminescence-based biosensors are a powerful tool to demonstrate induction of, and thus potential degradation by, specific pathways in situ. These biosensors can also distinguish the bioavailable from the inert contaminant because, by definition, they would only respond to bioavailable inducers. Biosensors are comprised of a receptor and the regulatory element (operator/promoter) that controls expression of the biodegradative operon, cloned upstream from the bioluminescence (*lux*) reporter functions. Thus, transcription of *lux* ensues when the biodegradative operon is induced. Biosensors have been developed to detect PAHs (Sayler 1998), PCBs (Layton et al. 1998), cadmium and lead (Tauriainen et al. 1996; Korpela et al. 1998). In all these biosensor strains, *lux* fusions have been developed, such as *nahG-lux*, *todCl-lux* and *bphA-lux*.

University of Delaware researchers have constructed a genetically engineered strain of *E. coli* that acts as a biosensor, glowing in the presence of certain kinds of chemical, including pesticides. The genetic material from bioluminescent bacteria was fused with *E. coli* in such a way that *E. coli* transcribed luminescent proteins under conditions of environmental stress. By adding these recombinant bacteria to poultry feed and then measuring the bioluminescent response, the scientists were able to detect a variety of compounds like 2,4-D and aflatoxin1, a carcinogen that kills chickens. Likewise, Zeneca, the British agrochemical manufacturer, is testing biochips that can spot dangerous pesticides and fungicides in the environment at an early stage. Such biosensors are expected to show whether an experimental pesticide would endanger humans, farm animals or plants. It could also help to show whether the chemicals are dangerous to bees and earthworms and how effective they are against pests.

Treating mixtures of organic and inorganic contaminants

Target pollutants rarely occur as sole contaminants in situ; rather they exist as components of complex mixtures of organic and inorganic compounds. The presence of heavy metals, which are not destroyed biologically but are only transformed from one oxidation state to another, interfere with the bioremediation processes. Genetic engineering allows transferring the heavy metal-resistance genes to known Gram-positive and Gram-negative hosts that can then serve as excellent raw material to construct recombinant strains to overcome this challenge. The strain *P. putida* Fl::mer (hybrid carrying benzene metabolism genes and mercury-resistance genes) was not only found to be resistant to high levels of both organic and inorganic species of mercury, but it also mineralized the benzene moiety split from phenylmercury (Horn et al. 1994). Recently, Crameri et al. (1997) demonstrated the modification of an arsenic-resistance operon, using DNA-shuffling. Cells expressing the optimized operon grew in up to 0.5 M arsenate, a 40-fold increase in resistance. Furthermore, a 12-fold increase in the activity of one of the gene products (a*rsC*) was observed in the absence of any physical modification to the gene itself. Such constructs could be useful for the treatment of high arsenate contaminated wastes and sites.

Biosurfactant applications to increase bioavailability

Biosurfactants are surface-active microbial products that have numerous industrial applications. Many microorganisms, especially bacteria, produce biosurfactants when grown on water-immiscible substrates. Most common biosurfactants are glycolipids in which carbohydrates are attached to a long-chain aliphatic acid, while others, like lipopeptides, lipoproteins and heteropolysaccharides, are more complex. The most promising appli-

cations of biosurfactants are in the cleaning of oil-contaminated tankers, oil-spill management, transportation of heavy crude, enhanced oil recovery, recovery of oil from sludge and bioremediation of sites contaminated with hydrocarbons, heavy metals and other pollutants. For details on the biosynthesis and applications of biosurfactants, excellent reviews are available (Desai and Banat 1997; Sullivan 1998).

Rhodococcus sp. 094 can oxidize alkanes in crude oil emulsified by nonionic chemical and biological surfactants (Bruheim et al. 1997). In *Acinetobacter radioresistens* KA53, one of the bioemulsifiers, alasan, was found to increase the solubility of PAHs by 6- to 27-fold (Rosenberg et al. 1998). The bioremediation of PAHs was designed by the addition of surfactants; and mathematical models were constructed to explain the effect of surfactants on biodegradation (Harayama 1997). With an increasing awareness of the applicability of biosurfactants, more research work is being focused on their utilization in the bioremediation of nonaqueous-phase lipids. One of the more recent reports is that of biosurfactant-enhanced hexadecane biodegradation by *P. aeruginosa* PG201 (Sekelsky and Shreve 1999).

The degradation of PCBs in the environment is mainly limited by their aqueous insolubility. Field application vectors were developed in which surfactants were used both to increase the solubility of the PCBs and to support the growth of surfactant-degrading strains engineered for PCB degradation (Lajoie et al. 1997). Given the inherent complexity of commercial surfactant preparations, the use of recombinant consortia to achieve extensive surfactant and PCB degradation appears to be an environmentally acceptable and effective PCB-remediation option. A rhamnolipid biosurfactant was found to solubilize hydrophobic α-HCH in water and promote HCH degradation by a *Pseudomonas* Ptm^+ strain (Karanth and Ramesh 1998). It was also found that, during bioremediation of HCH contaminated soil using this bacterium, in vivo genetic recombination occurred, resulting in enhanced biodegradation in soil. The genes involved in biosurfactant synthesis have been characterized and the molecular genetics of regulatory mechanisms of biosurfactant production have been studied in detail (Sullivan 1998).

Conclusions

The potential of microorganisms in the remediation of some of the compounds hitherto known to be undegradable has been widely acknowledged globally. Recent advances in the molecular genetics of degradation and studies on enzyme-tailoring and DNA-shuffling are discussed above. With advances in biotechnology, bioremediation has become a rapidly growing area and has been commercially applied for the treatment of hazardous wastes and contaminated sites. A center and a database have been established on biocatalysis and biodegradation (http://umbbd.ahc.umn.edu). The database contains information on microbial biocatalytic reactions and biodegradation pathways primarily for xenobiotics. The goal of the database is to provide information on microbial enzyme-catalyzed reactions that are important for biotechnology.

Although a wide range of new microorganisms have been discovered that are able to degrade highly stable, toxic organic xenobiotics, still many pollutants persist in the environment. A number of reasons have been identified as challenges posed to the microorganisms working in contaminated sites. Such potential limitations to biological treatments include: poor bioavailability of chemicals, presence of other toxic compounds, inadequate supply of nutrients and insufficient biochemical potential for effective biodegradation. A wide range of bioremediation strategies have been developed for the treatment of contaminated soils using natural and modified microorganisms. Selecting the most appropriate strategy to treat a specific site can be guided by considering three basic principles: the amenability of the pollutant to biological transformation to less toxic products, the bioavailability of the contaminant to microorganisms and the opportunity for bioprocess optimization. With the help of advances in bioinformatics, biotechnology holds a bright future for developing bioprocesses for environmental applications.

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