Engineering bacteria for bioremediation

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The treatment of environmental pollution by microorganisms is a promising technology. Various genetic approaches have been developed and used to optimize the enzymes, metabolic pathways and organisms relevant for biodegradation. New information on the metabolic routes and bottlenecks of degradation is still accumulating, enlarging the available toolbox. With molecular methods allowing the characterization of microbial community structure and activities, the performance of microorganisms under *in situ* conditions and in concert with the indigenous microflora will become predictable.

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Abbreviations

PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
TNT	trinitrotoluene

Introduction

The use of microbial metabolic potential for eliminating environmental pollutants provides a safe and economic alternative to their disposal in waste dump sites and to commonly used physico-chemical strategies. Microorganisms capable of mineralizing a variety of toxic compounds under laboratory conditions have been isolated. The accumulation in the environment of highly toxic and persistent compounds, however, emphasizes the fact that the natural metabolic diversity of the autochthonous microbes is insufficient to protect the biosphere from anthropogenic pollution.

Many recalcitrant chemicals contain structural elements or substituents that do not (or seldom) occur in nature (xenobiotics). Presumably, because of the novelty of these compounds, microorganisms have not evolved appropriate metabolic pathways for them. Whereas for some xenobiotics no degradative routes have been described, others are transformed incompletely or inefficiently, or the complex mixtures of contaminants prevent degradation by existing pathways. A solely biochemical explanation is not sufficient, however, for explaining the accumulation of such compounds. Efficient degradation involves various factors, such as bioavailability of the substrates, which have to be understood. Furthermore, the substrate has to diffuse or be transported into the cell. Besides these factors, the organism capable of degradation must be present at the site where it is needed and it has to perform under the given or manipulated environmental conditions.

Thus, a combined approach is required to understand the bottlenecks of xenobiotic degradation, to rationally overcome them by different (genetic) engineering methods, to elucidate the microbial metabolic diversity and to understand the metabolic and organismic network necessary for activity under environmental conditions.

In this review, we will discuss the advances in understanding the natural diversity and capabilities of microorganisms for degrading aromatic and xenobiotic organic compounds, the engineering of enzymes, catabolic pathways and organisms for bioremediation purposes, and methods for characterizing microbial activities *in situ*.

Classical analysis and exploitation of the natural biodiversity

New information on metabolic pathways

Naturally occurring microbial activities are and have been the starting point for all biotechnological applications. It is therefore necessary to isolate bacterial strains with novel metabolic capabilities and to biochemically and genetically elucidate degradative pathways.

Aromatic compounds are usually activated for subsequent reactions by the introduction of two hydroxyl-groups, either in *ortho*- or *para*-position to one another, which in the case of hydrophobic aromatics is usually achieved by multi-component dioxygenases, composed of an electron transport chain and the catalytically active α- and β-subunits. These enzymes usually define the range of substrates that can be transformed by a certain metabolic pathway, and their diversity and substrate ranges, therefore, awaited major attention. Serious advances have been made on the biochemical and genetic characterization of such enzymes and their respective genes. Dioxin dioxygenase of *Sphingomonas* sp. strain RW1 was among the first enzymes reported to be capable of carrying out an angular dioxygenation, that is, oxygenation at a pair of vicinal carbon atoms, one of which is involved in one of the bridges between the two aromatic rings. The respective genes are now characterized and whereas genes coding for multi-component dioxygenases are usually clustered, those coding for dioxin dioxygenase were unexpectedly scattered throughout the chromosome [1•]. A second recently characterized multi-component enzyme capable of carrying out angular dioxygenation is carbazole 1,9a dioxygenase, the terminal oxygenase of which consists of a single protein CarAa, contrasting with the classical oxygenase composition of large α and β subunits. The nucleotide and deduced amino acid sequences of CarAa are, again, unique and exhibit only poor similarities with

other large subunits of terminal oxygenases, including dioxin dioxygenase [2]. The broad substrate range of the gene product has now been reported [3]. Another relatively recently identified lineage of ring-activating dioxygenases has been characterized genetically [4•] to contain, between the genes coding for the electron transport chain of a naphthalene dioxygenase, genes coding for subunits of an enzyme catalyzing a later step in the pathway, that is, salicylate 5-hydroxylase. Even other relatively unrelated naphthalene and phenanthrene systems from *Rhodococcus* sp. NCIMB 12038 [5] and *Burkholderia* sp. strain RP007 [6] have been described, but no substrate specificity profiles have been given. Such results indicate, however, the broad diversity of ring-activating dioxygenases and allows one to assume that new genes with new specificities wait to be discovered.

Although polyaromatic hydrocarbon (PAH) degradation has been relatively well characterized in terrestrial isolates, little was known about the mechanisms by which marine bacteria catabolize such compounds. New genera capable of PAH degradation, such as *Cytoclasticus* [7] and *Neptunomonas* [8], have now been isolated. The respective genes were shown to be distantly related to the genes encoding naphthalene dioxygenases of *Pseudomonas* and *Burkholderia* strains and thus form subgroups in the *nah* gene family.

Advances in understanding bacterial degradative diversity are not restricted to the initial ring-activating dioxygenases but also apply to enzymes dealing with metabolites of aromatic degradation. Aromatic ring-cleavage can be regarded as a major key reaction in haloaromatic degradation, and mineralization has been reported, with a few exceptions, only after intradiol cleavage of chlorocatechols or chlorohydroxyhydroquinones. It was assumed for a long time that it was impossible to metabolize 3-chlorinated catechols via the *meta*-cleavage pathway, because the reaction products would inactivate the extradiol dioxygenase. *Pseudomonas putida* strain GJ31 containing a novel chlorocatechol 2,3-dioxygenase that can efficiently cleave 3-chlorocatechol at the 2,3-position, leading to simultaneous ring-cleavage and dechlorination, thereby allowing the strain to degrade chlorobenzene via a *meta*-cleavage pathway, has recently been described [9], however, and residues responsible for resistance to suicide inactivation were localized [10]. A similar type of reaction is also assumed to occur in the degradation of pentachlorophenol and γ-hexachlorocyclohexane (lindane) [11•–13•].

Another new type of *meta*-cleavage dioxygenase has recently been reported to be involved in the degradation of various nitroaromatics [14,15]. Whereas the ring-cleavage substrates are normally diphenols with the two hydroxy groups either *ortho* or *para* to each other, in the case reported, only one hydroxyl-group is present in the ring-cleavage substrate 2-aminophenol, which undergoes ring-cleavage to 2-aminomuconic semialdehyde. The amino group obviously substitutes for a second hydroxyl function.

Bioavailability of xenobiotics

One of the main reasons for the prolonged persistence of hydrophobic organic compounds in the environment is their solubilization-limited bioavailability. A possible way to enhance their bioavailability and, thereby, their biodegradation is the application of (bio)surfactants, molecules that consist of both a hydrophilic and hydrophobic part, and which in most of the studies reported thus for have been introduced seperately. Reports on the efficacy of surfactants on bioremediation have, however, been mixed. The natural roles of biosurfactants have been claimed to increase the surface area of hydrophobic, water-insoluble growth substrates, increasing their bioavailability by increasing the apparent solubility or desorbing them from surfaces and regulating attachment and detachment of microorganisms to and from surfaces [16]. Thus, the net effect of a surfactant on biodegradation depends on the benefits that result from enhanced solubility of target compounds versus the reduction in direct adhesion of bacteria to those compounds. Stelmack *et al.* [17] showed that the addition of surfactants reduced bacterial adhesion to the surfaces of non-aqueous phase liquids and, concomitantly, growth on anthracene. Thus, contrasting effects of surfactant application are a result of the poorly understood complexity of interactions between soil/sediment, pollutant, surfactant and microorganisms in different environments. The recent observations that single surfactants can have contrasting effects on the degradation of organic pollutants [18•] may further explain why applications of surfactants have yielded inconclusive results. There is certainly a need to design an optimal surfactant/biodegrader/target environment combination and to further unravel the underlying complex interactions. Thus, although with the current knowledge the optimization of degradation by unknown metabolic communities on site through the addition of surfactants remains a trial and error test, optimization of defined pure or mixed cultures can be performed. The combination of surfactant production with degradative capabilities in a single bacterial strain [19•] will offer advances for *in situ* bioremediation, but further insights into the genetic organization and regulation of surfactant production are needed (for a review see [20]).

Transport and chemotaxis

Many aromatic compounds are taken up by bacteria through energy-dependent transport systems. Pao *et al.* [21•] recently listed 18 transport protein families within the major facilitator superfamily. The narrow specificity of such permeases has been shown recently, for example, for phthalate permease [22]. There is now accumulating information that xenobiotic compounds are also transported by specialized transporter systems. A transporter for 2,4-dichlorophenoxyacetate has been reported initially by Leveau *et al.* [23]. Enantioselective uptake was shown for the chiral, similarly-structured herbicide 2-(2,4-dichlorophenoxy)propionate [24•]. Whitman *et al.* [25] even gave evidence for the presence of an active transport system in *Pseudomonas fluorescens* for the noncharged,

hydrophobic naphthalene molecule. Clearly, transport mechanisms have to be taken into consideration when designing superior biocatalysts for bioremediation purposes.

The 4-hydroxybenzoate transporter PcaK of *P. putida* is also responsible for chemotaxis to this compound. It is, to date, the only major facilitator superfamily transporter involved in chemoreception and, thus, is different from the described classical chemoreceptors [26]. Grimm and Harwood [27•] reported that the plasmid-encoded membrane protein NahY is required for chemotaxis with naphthalene. Its carboxy-terminal region resembles chemotaxis transducer proteins, thereby functioning as a chemoreceptor for naphthalene and, possibly, for related compounds, such as biphenyl [28]. Chemotaxis towards pollutants may enhance their biodegradation in natural environments. The understanding of the genetic basis for chemotaxis will enable the rational use of such genetic determinants.

Properties of organisms important for bioremediation

Various environmental contaminants, such as toluene, are highly hydrophobic. They are toxic for microorganisms because they accumulate in and disrupt cell membranes, inactivate the cells and thereby abolish the desired biodegradative activity, even in microorganisms capable of biodegradation. Several bacteria resistant to solvents have been isolated and possible mechanisms of organic solvent tolerance, such as alterations in the composition of the cytoplasmic and outer membranes, as well as the cell surface, have been reported (for a recent review see [29]). The *cis* to *trans* isomerization of fatty acids is one of the adaptive mechanisms. Because of the higher rigidity of *trans* fatty acids, the membrane is less susceptible to the structural disturbances caused by the organic solvent. The gene encoding an enzyme responsible for the *cis* to *trans* isomerization of fatty acids has now been cloned and characterized [30]. An increased biosynthesis of phospholipids has also been observed in solvent-tolerant microorganisms. Studies comparing the solvent-tolerant wild type with solvent-sensitive mutants have shown that low cell-surface hydrophobicity (modification of the lipopolysaccharide or porines of the outer membrane) serves as a defense mechanism that prevents the accumulation of organic solvent molecules in the membrane [31]. In addition to these adaptive changes, active mechanisms such as the presence of solvent efflux pump systems, which are often linked to multidrug efflux pump systems [32,33], contribute to organic solvent tolerance. The elimination of higher solvent concentrations through its effective degradation was found to be not responsible for solvent tolerance [34]. Nevertheless, some of the isolated solvent-tolerant bacteria are also capable of mineralization of, for example, toluene, and the catabolic potential can be engineered to include substrates previously not mineralizable by the given organism [35]. As evidenced by Huertas *et al.* [36], in sites heavily polluted by aromatic hydrocarbons, solvent-tolerant strains would be expected to become established first, to colonize the site, and to become predominant in the removal of these pollutants. Thus, equipping solvent-tolerant bacteria with an appropriate catabolic potential will be a promising approach for bioremediation purposes.

A similar approach to equip bacteria adapted to a certain environment with a new catabolic potential was used by Lange *et al.* [37], who constructed a recombinant *Deinococcus radiodurans* capable of oxidizing toluene and chlorobenzene in highly irradiating environments. Other extreme environments that require remediation include Arctic and Antarctic sites. Polychlorinated biphenyl (PCB)-degrading psychrotolerant bacteria have recently been isolated [38]. Even though the results suggest that the respective enzymes are cold-adapted, it remains to be proven whether this is actually the case or if the cell membrane composition facilitates transport at low temperatures. Nevertheless, either the organisms themselves or the enzymes and information acquired thereof can, without doubt, help in optimizing future bioremediation efforts.

Changes in cell-surface hydrophobicity are not only reported to be a defense mechanism against organic solvents, but also to be involved in the adhesion of bacteria to surfaces [39]. As for bioremediation purposes, dispersion of inoculant cells relative to their point of introduction is desirable and blocking of wells should be avoided; therefore, adhesion-deficient strains could be advantageous and, in fact, appropriate mutant strains which are rapidly transported through soils recently have been produced [40•].

New developments on organisms capable of enhanced biodegradation

Optimizing bacteria and transgenic plants

One strategy for designing superior biocatalysts is the rational combination of catabolic segments from different organisms within one recipient strain. Thereby, complete metabolic routes for xenobiotics, which are only co-metabolized, can be generated and the formation of dead-end products or even toxic metabolites can be avoided. This strategy has been applied successfully for the degradation of highly toxic trihalopropanes, for which mineralization has not yet been described [41•].

A similar strategy of combining complementary metabolic activities can be used for the development of microorganisms capable of mineralizing PCBs by combining an oxidative pathway for (chloro)biphenyl transformation (encoded by the *bph* genes) into (chloro)benzoate with a chlorobenzoate degradative pathway. Several hybrid strains have been engineered in recent years by conjugative matings [42•] of appropriate organisms or by introduction of the *bph* genes into chlorobenzoate degraders, usually using a degradative pathway for chlorobenzoates via the corresponding chlorocatechols. By cloning and expressing the genes encoding enzymes for *ortho*- and *para*-dechlorination of chlorobenzoates in the biphenyl-degrading and chlorinated biphenyls co-metabolizing strain *Comamonas testosteroni* strain VP44, derivatives

capable of growing on and completely dechlorinating 2- and 4-chlorobiphenyl were obtained [43•].

Metabolic routes or catabolic fragments can be assembled or introduced not only into appropriate bacterial recipient strains. Accumulation of heavy metals by plants is a well-characterized technology for bioremediation, and plants can be supplemented with additional genetic information of bacterial origin. Transgenic poplar plantlets expressing bacterial mercuric reductase were shown to germinate and grow in the presence of levels of ionic mercury that are normally toxic [44] and to release elemental mercury, thereby transporting soil-bound mercury efficiently out of the soil. *Arabidopsis thaliana* was engineered to express a modified organomercurial lyase [45] and those transgenic plants grew vigorously on a wide range of concentrations of highly toxic organomercurials, probably by forming ionic mercury, which should accumulate in the disposable plant tissues.

French *et al.* [46••] gave the first report of genetically modified plants for the transformation of xenobiotic contaminants to nontoxic material. They previously reported that *Enterobacter cloacae* PB2 is capable of growth with trinitrotoluene (TNT) as a nitrogen source. The pentaerythritol tetranitrate reductase, an enzyme described to be involved in the degradation of nitrate esters, is capable of reducing the aromatic ring of TNT and causing liberation of nitrite [46••,47]. Unfortunately, the final transformation products have not yet been characterized, however, toxic products containing reduced nitrofunctions were clearly not formed. Seeds from transgenic tobacco plants expressing pentaeryhritol tetranitrate reductase were able to germinate and grow at concentrations of glycerol trinitrate or TNT that inhibited the germination and growth of wild-type seeds. Seedlings also showed a more rapid and complete denitration of glycerol trinitrate than did wild-type seedlings. Transgenic plants can, therefore, become an alternative to optimized bacteria for bioremediation purposes.

Molecular tools

A general problem of using recombinant strains in bioremediation is the instability of the cloned genes when borne on plasmids and the inheritance of marker genes used for selection. The problem of stability has been overcome by the use of so-called mini-transposons for the stable integration of genes into the chromosome of recipient strains (for a review see [48]). The usefulness of the mini-transposon method has recently been evidenced again by the construction of highly stable recombinant strains carrying genetic expression cassettes with different oxygenase-encoding genes on their chromosomes [49]. Antibiotic-resistance markers were replaced by non-antibiotic markers, such as the easy-to-use tellurite-resistance determinants [50]. An elegant method is the deletion of all unnecessary recombinant tags inherited from previous cloning steps, resulting in quasi-natural strains bearing exclusively the DNA segment encoding the phenotype of choice [51•].

Optimizing biocatalysts

The increasing information on the structure and function of catabolic enzymes and pathways offers further possibilities for their optimization. A rational site-directed mutagenesis approach to improving enzyme function is possible if a detailed characterization of a given enzyme and at best the crystal structure is available. Alternatively, sequence alignments can help in identifying residues critical for enzyme activity (usually highly conserved) or substrate specificity (usually visible as differences in the sequences), and a rational design can lead to improved biocatalysts. Besides such site-directed approaches, various DNA-shuffling methods (i.e. the random fragmentation of a population of mutant genes of a certain family followed by random reassembly) have been developed, which allow the creation of a vast range of chimeric proteins and protein variants. However, useful methods for screening the variety of derivatives are available only in some cases, for example, when the desired reaction can be coupled to an obvious phenotype or color reaction.

Haloalkane dehalogenases were among the first enzymes in xenobiotic degradation where, based on crystal structure information, enzymes with higher catalytic activity could be created [52,53]. To understand the specificity of different haloalkane dehalogenases in detail, protein sequences and models of tertiary structures of haloalkane dehalogenases have now been compared and functionally important amino acids, as possible targets for future site-directed mutagenesis experiments, were predicted [54]. Based on known three-dimensional structures, Vollmer *et al.* [55•] constructed variants of a muconate cycloisomerase (involved in the degradation of natural aromatic compounds) containing amino acids found in equivalent positions in the binding cavity of chloromuconate cycloisomerases (involved in the degradation of chloroaromatics) and could increase the specificity constants for some chloromuconates. In various other aspects, however, the mutant enzymes retained wild-type characteristics. It became evident that evolution from muconate to chloromuconate cycloisomerase was a rather complex process. Rather than simple changes in the binding cavity, other or more complex changes are thus responsible for the observed differences between the enzymes. A similar complexity of residue importance at the active site was observed when analyzing extradiol dioxygenases. An enzyme, superior to most other extradiol dioxygenases by its ability to oxidize 3-chlorocatechol in a distal manner, lost its activity much more rapidly during oxidation of various substrates. The results of site-directed mutagenesis studies [56•] show that optimization of the catalytic performance of this enzyme, in one respect, leads to worsening in another. Detailed analysis of mutant enzymes is, therefore, necessary to evaluate critically the effectiveness of engineered derivatives.

In cases where structural information is not available, amino acid sequence comparisons between enzymes can

give indications of the residues important for catalytic activity. Information on the regions of an enzyme involved in determination of substrate specificity can be obtained by creation of hybrids between related enzymes with different substrate specificity or catalytic properties. This strategy has been applied to determine the residues responsible for the difference in substrate specificity of biphenyl dioxygenases [57]. Based upon the results, site-directed mutagenesis studies were performed that resulted in the expansion of the range of PCB congeners biodegradable by a single enzyme. Parales *et al.* [58] showed that, as observed for biphenyl dioxygenases, the carboxy-terminal region of the large subunit of the oxygenase component was responsible for the differences in enzyme specificity observed between 2-nitrotoluene and 2,4-dinitrotoluene dioxygenases. In contrast, hybrid enzymes created from a toluene and chlorobenzene dioxygenase indicated that regions that determine the substrate specificity are different from those previously identified in biphenyl dioxygenases, and a single amino acid near the catalytic site was shown to be crucial for substrate specificity [59•].

The three-dimensional structures of biphenyl or toluene dioxygenases are not known at present; however, the structure of a similar naphthalene dioxygenase was recently elucidated [60•]. It was suggested that residues shown to be important for substrate specificities of biphenyl dioxygenases align structurally with residues in naphthalene dioxygenase that interact directly with hydrophobic substrates or those located near the narrow gorge, which provide substrates with access to the iron ion [61]. Further analysis of biphenyl dioxygenase derivatives indicated that changes outside of regions reported to be critical can affect the specificity when occurring in concert with appropriate changes within the critical regions [61]. These results clearly indicate that a broad range of different hybrid enzymes must be analyzed.

Methods applying intensive mutagenesis in combination with shuffling of the generated mutations will result in a large library of genes with different mutations [62]. Family shuffling (i.e. shuffling of naturally occurring homologous sequences) assumes that chimeric analogs derived from various homologous proteins could gain favorable properties, as has been shown for the large subunits of biphenyl dioxygenases [63]. Family shuffling methods have been refined excluding the preferential reformation of non-hybrid molecules and leading to an approximately quantitative formation of hybrid enzymes [64]. As well, the broad natural diversity of enzymes can be analyzed and recruited, without the need to isolate the appropriate organisms or enzymes. This was performed by isolating from the environment the central coding segments of the genes of choice through the use of degenerate PCR primers designed from amino acid sequences conserved among the class of enzymes analyzed (in this case extradiol cleavage dioxygenases) and flanking the central gene segments and inserting them into the flanking regions of a related gene [65••]. Heat-resistant hybrids of catechol 2,3-dioxygenase enzymes could be easily detected due to the bright yellow color of the reaction product formed even at elevated temeratures. This color reaction was also the basis for screening optimized biphenyl dioxygenases [61]. Yellow ring-cleavage products can, however, only be formed when the dioxygenation product is further transformed by the two subsequent pathway enzymes and thus only if those two enzymes do not constitute a pathway bottleneck. Lin *et al.* [66] generated a variant of horseradish peroxidase that is expressed in an active form in *Escherichia coli*. The enzyme is used for the coupling of phenolic products of aromatic substrates to generate colored or fluorescent compounds and co-expression creates a pathway for the conversion of aromatic substrates into easily detectable compounds *in vivo* [67]. Using this system, variants of cytochrome P450cam with high activity against naphthalene could be identified easily [68•]. The system could also be used to detect catechols formed from benzene derivatives by the concerted action of a toluene dioxygenase and the subsequent pathway enzyme toluene dihydrodiol dehydrogenase [67]. Despite its superiority compared to previously developed tests, the system still depends on the presence of a toluene dihydrodiol dehydrogenase that exhibits a sufficiently broad substrate specifically to further transform all dioxygenation products into the respective catechols.

Tools to characterize and follow active organisms in the environment

Attempts to demonstrate the potential for bioaugmentation in soils have resulted in successes and failures. An understanding is necessary of the bioavailability of a pollutant, of the survival, activity and transport of added microorganisms or their genetic material, and on the general environmental conditions. Furthermore, the intrinsic capabilities and activities of the site of interest have to be characterized, on one hand, to suggest the bottlenecks of biodegradation and, on the other hand, possibly to recruit the intrinsic potential for biotechnological applications. Genetic engineering techniques offer the possibility to equip organisms, known to survive and be active when introduced into certain environments, with the desired catabolic potential [69].

PCR methods offer a sensitive method to follow introduced bacteria in the environment. Both the introduced microorganism and the introduced catabolic genes can be monitored and even quantified [70]. The same strategies of PCR amplification of genes coding for degradative enzymes and DNA:DNA hybridizations using PCR-generated labeled gene probes, can be used to characterize a certain environment concerning its metabolic potential or concerning the metabolic pathway predominant in a certain environment [71,72]. Extraction and characterization of mRNA provides an indication of the activity and can be used to identify those genes of predominant importance at the site under study [73••]. By comparing the results of culture-independent methods (i.e. the analysis of 16S ribosomal DNA fragments

and genes coding for a phenol hydroxylase) with those obtained by different enrichment strategies, Watanabe *et al.* [74•] could identify the dominant phenol-degrading populations in the sludge analyzed and show that they could be isolated by direct plating or chemostat enrichment, but not by classical batch enrichment. That batch enrichments produce different results from those of continuous culture enrichments was further evidenced by the analysis of a 4-chlorosalicylate degrading chemostat community [75]. Whereas monochlorinated aromatics are usually degraded by organisms isolated thus far via chlorocatechol and a subsequent respectively modified *ortho*-cleavage pathway, the chemostat community comprised organisms with a new pathway for chloroaromatic degredation which uses enzymes of the 'classical' *ortho*-cleavage (3-oxoadipate) pathway. The identification of new degradative pathways demonstrates that the biodegradative potential of microorganisms is not yet adequately understood. New enrichment strategies will aid in making available the broad diversity of microorganisms, which can then be exploited and optimized for biotechnological applications. New methods available for the characterization of the active members in microbial communities, such as incorporation of isotope markers into the taxonomically relevant phospholipid fatty acids [76] or combination of fluorescence *in situ* hybridization for visualization of specific groups of bacteria with microautoradiography for visualization of active members [77,78••], will allow the adaptation of enrichment strategies for important species.

Conclusions

It is evident that we are just beginning to understand and, thus, to fully exploit the natural diversity for biodegradation and bioremediation purposes. New genes, enzymes and metabolic routes involved in bacterial xenobiotic degradation have been discovered, and new methods have been developed, which allow the discovery of the broad flexibility of microorganisms. Besides application of the natural diversity, the artificial evolution of enzymes and pathways will lead, without doubt, to improved biocatalysts and high-throughput methods of screening for the desired phenotypes are becoming available. Strategies to design superior biocatalysts are taking into account more and more the necessity of such organisms to perform in a reliable fashion under environmental conditions. Thus, studies to understand the interaction between xenobiotics and organisms and on the fate, survival and activities of microorganisms in the environment have to intersect with the biochemical and genetic engineering studies. Such a crossfeeding will provide the ground for successful interventions into environmental processes and, thereby, lead to optimized strategies for bioremediation.

Update

Evidently, the sequence diversity in genes for the degradation of aromatic compounds is rather broad and new genes and genetic organizations are still being discovered. The catabolic genes of a *Nocardioides* strain capable of degrading phenanthrene but not naphthalene have now been described [79]. The genetic organization of the genes encoding phenanthrene dioxygenase, the phylogenetically diverged positions of these genes and an unusual type of ferredoxin component suggest this enzyme to be a new class of aromatic-ring-hydroxylating dioxygenases. It was thus far assumed that resistance to hydrophobic pollutants is not linked to the degradative capabilities. Mosqueda and Ramos [80] have now shown that a second toluene efflux system is actually linked to the *tod* genes for toluene metabolism in *P. putida* DOT-T1E. The rational combination of a degradative pathway with genes encoding for functions of possible potential for enhancing bioremediation has been carried out by combination of 2,4-dinitrotoluene degradation with the production of a prokaryotic hemoglobin [81]. Recombinant *Burkholderia* stains expressing the hemoglobin grew faster on dinitrotoluene than the wild type. It has still to be elucidated if those recombinants have an improved ability to bioremediate dinitrotoluene, especially in the presence of low oxygen concentrations. Engineering of phytodetoxification of organomercurials has been extended by simultaneous introduction of two genes encoding mercuric reductase and organomercurial lyase, thus enabling plants to transform organic mercury into volatile and less toxic elemental mercury [82].

The optimization of biocatalysts by rational site-directed mutagenesis based on available crystal structures was initiated for naphthalene dioxygenase [83], where the crystal structure had just recently become available and extended for cytochrome P450cam [84]. New cytochrome P450cam variants with elevated phenanthrene and fluoranthrene oxidation rates could be engineered.

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