Available online at www.sciencedirect.com
SCIENCE DIRECT
Enzyme and Microbial Technology 37 (2005) 487–496
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Recent advances in the bioremediation of persistent organic pollutants via biomolecular engineering

Ee Lui Ang [a,b], Huimin Zhao [a,*], Jeffrey P. Obbard [b,**]

[a] *Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, 600 S. Mathews Ave, Urbana, IL 61801, USA*
[b] *Department of Chemical and Biomolecular Engineering, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*

Received 24 January 2004; received in revised form 17 July 2004; accepted 17 July 2004

Abstract

With recent advances in biomolecular engineering, the bioremediation of persistent organic pollutants (POPs) using genetically modified microorganisms has become a rapidly growing area of research for environmental protection. Two main biomolecular approaches, rational design and directed evolution, have been developed to engineer enhanced microorganisms and enzymes for the biodegradation of POPs. This review describes the most recent developments and applications of these biomolecular tools for enhancing the capability of microorganisms to bioremediate three major classes of POPs – polycyclic aromatic hydrocabons (PAHs), polychlorinated biphenyls (PCBs) and pesticides. Most of the examples focused on the redesign of various features of the enzymes involved in the bioremediation of POPs, including the enzyme expression level, enzymatic activity and substrate specificity. Overall, the rapidly expanding potential of biomolecular engineering techniques has created the exciting potential of remediating some of the most recalcitrant and hazardous compounds in the environment.

Keywords: Persistent organic pollutants; Biomolecular engineering; Bioremediation; Directed evolution

1. Introduction

In tandem with rapid industrial and economic development, human activities have instigated widespread pollution of the natural global environment. Anthropogenic organic pollutants are now dispersed throughout the environment and can be highly recalcitrant to biodegradation processes found in naturally occurring microorganisms. Due to their potential toxicity to both wildlife and humans, several persistent organic pollutants (POPs) have now been totally banned from production and use in many countries around the world. In 2001, the Stockholm Convention under the auspices of United Nation Environmental Program, specified a suite of POPs considered as potential endocrine disrupting chemicals (EDCs) in the environment [1]. Such concerns have heightened the need for novel and advanced bioremediation techniques to effectively remove POPs from a variety of contaminated environmental media including water, sediments and soils [2,3].

Bioremediation has distinct advantages over physicochemical remediation methods as it can be cost-effective and achieve the complete degradation of organic pollutants without collateral destruction of the site material or its indigenous flora and fauna [4]. However, the acquisition of biodegradative capabilities by native microorganisms at contaminated sites through natural evolutionary processes such as random mutation often occur at an unacceptably slow rate, particularly when multiple biodegradation traits are required—as is the case of with sites cocontaminated with more than one organic compound. Within this context, accelerating these evolutionary processes via biomolecular engineering tech-

Abbreviations: Bph, biphenyl dioxygenase; CB, chlorobiphenyls; CBA, (chloro)benzoate; CYP, cytochrome P450 monooxygenase; GEM, genetically engineered microorganism; IVC, in vitro compartmentalization; MGE, mobile genetic element; NADP, nicotinamide adenine dinucleotide phosphate; OP, organophosphates; OPH, organophosphorus hydrolase; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; POP, persistent organic pollutant; PTE, phosphotriesterase

* Corresponding author. Tel.: +1 217 333 2631; fax: +1 217 333 5052.
** Corresponding author. Tel.: +65 6874 2884; fax: +65 6872 5483.
E-mail addresses: zhao5@uiuc.edu (H. Zhao), chejpo@nus.edu.sg (J.P. Obbard).

0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2004.07.024

niques has become an increasingly attractive proposition for enhanced bioremediation strategies.

The objective of this review is to highlight and evaluate the most recent developments in biomolecular engineering for enhancing the biocatalytic capability of microorganisms to biodegrade POPs. The review focuses on the application of biomolecular techniques to the bioremediation of three major classes of POPs, i.e. polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and pesticides. Biomolecular engineering holds opportunities for the rapid advancement of bioremediation technology and offers the prospect of degrading some of the most recalcitrant and toxic xenobiotic POPs in the modern global environment.

2. Tools for biomolecular engineering

Biomolecular engineering is a relatively new field of academic research and industrial practice with a goal of engineering biomolecules, such as proteins and nucleic acids, and biomolecular processes for biotechnological applications [5]. In recent years, two different, yet complementary strategies in biomolecular engineering have been developed to genetically engineer enzymes or microorganisms for bioremediation applications: rational design and directed evolution (Fig. 1).

The rational design approach for bioremediation typically involves either the construction of a single microorganism in which desirable biodegradation pathways or enzymes from different organisms are brought together to perform specific reactions using recombinant DNA technology (whole cell level), or the engineering of enzymes with desired characteristics using site-directed mutagenesis (protein level). Enzymes are delicately folded proteins, where even small changes in the amino acid sequence can disrupt the protein configuration and diminish catalytic activities. Moreover, it is near impossible to predict the impact of a modification in a single trait of the enzyme on other biochemical properties. Thus, to successfully modify an enzyme using rational design, a huge amount of a priori information on the structural, mechanistic and dynamic properties of the protein is required. This places an enormous demand on manpower and laboratory resources. Nonetheless, the capability of rational design is rapidly improving due to recent advances in enabling technologies such as X-ray crystallography and bioinformatics.

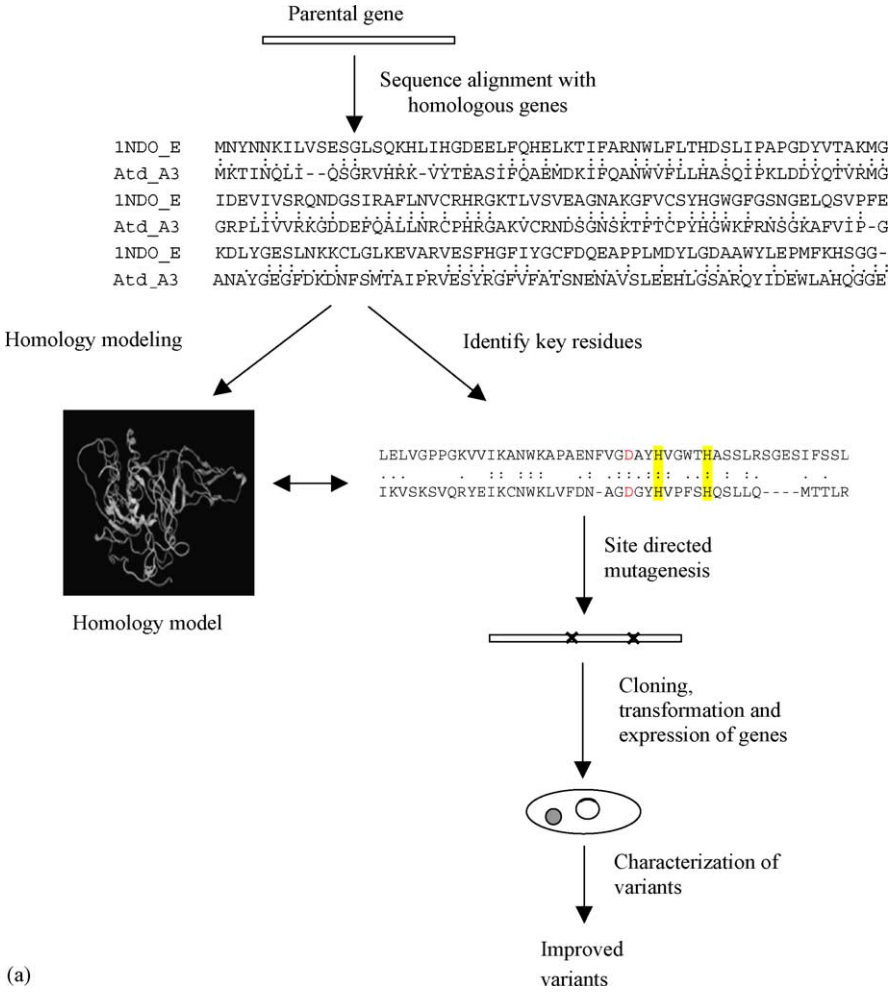

Fig. 1. Generalized scheme of (a) the rational design process and (b) the directed evolution process.

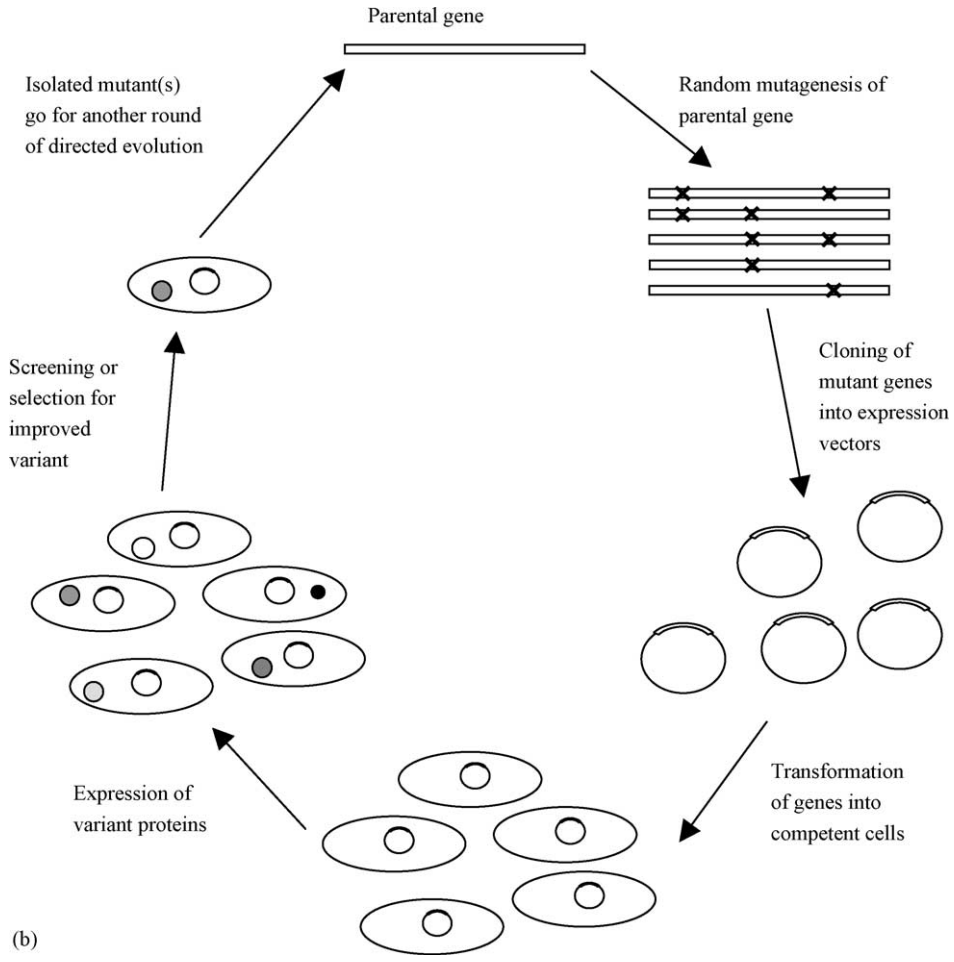

Fig. 1. (*Continued*).

In contrast to rational design, directed evolution does not require a priori knowledge of the protein structure and can identify mutations that influence enzyme activity through subtle long-range interactions. This approach mimics a simple algorithm that nature has successfully used over eons of time, i.e. genetic diversification coupled with natural selection pressure [6,7]. However, unlike natural evolution, directed evolution has a specific goal that is empirically controlled in the laboratory, and can collapse the process into a matter of months or even weeks. In essence, directed evolution involves the creation of a diverse library of gene variants through random mutagenesis, such as error prone PCR or gene recombination techniques, such as the in vitro staggered extension process (StEP) recombination [8] and in vivo DNA shuffling [9], followed by selection or screening, to obtain the enzymes or pathways with the desired characteristics. The process is iterative where the selected or screened enzymes are subjected to further rounds of random mutagenesis or gene recombination to produce a new generation of enzyme or biochemical pathway variants in a microorganism. For the bioremediation of POPs, this means the generation of a genetically capable organism or enzyme for the complete biodegradation of the compound of interest.

3. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are aromatic compounds made up of two or more fused benzene rings. PAHs are recalcitrant and can persist in the environment for long periods, but are conducive to biodegradation by certain enzymes found in bacteria and fungi [10]. In the past several years, several oxidoreductases such as laccases and cytochrome P450 monooxygenases (CYPs) have been exploited for the enzymatic degradation of PAHs.

Laccases belong to a group of multicopper enzymes that can catalyze the oxidation of a wide variety of phenolic compounds including PAHs. Like other ligninolytic enzymes, laccase is difficult to express in non-fungal systems and knowledge of structure–function relations underlying the key functional properties of laccase is limited. Hence, directed evolution holds exciting potential for improving the performance of the enzyme. In a study undertaken by Bulter et al. [11], the laccase gene from *Myceliophthora thermophia* (*MtL*), which was previously expressed only in *Asperigillus oryzae*, was transformed into *Saccharomyces cerevisiae* and subjected to directed evolution. After 10 rounds of directed evolution, a mutant with a 8-fold increase in laccase ex-

pression and a 22-fold increase in the k_{cat} for 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was created. The final mutant had a total activity 170-fold higher than the wild type. The MtL enzyme holds great potential for the bioremediation of PAHs due to its high thermal stability that enables it to work at the elevated temperatures needed to increase the solubility of highly recalcitrant PAHs. However, this particular enzyme has yet to be tested on actual PAH degradation. It is noteworthy that, although laccases are promising catalysts with a variety of potential uses, their applications have been limited by their requirement for mediators. Thus, engineering of a laccase with activity in the absence of mediators would be a suitable target for directed evolution.

CYPs are one of the largest known enzyme superfamilies and are expressed in most living organisms. PAHs can be oxidized by CYP enzymes to form catechols, which are then degraded by other enzymes, including catechol dioxygenases to harmless products and incorporated into the tricarboxylic acid cycle of microorganisms. Wild-type CYP101 ($P450_{cam}$) from *Pseudomonas putida* has been shown to have an inherently low activity (<0.01 min^{-1}) towards the PAH substrates phenanthrene, fluoranthene, pyrene and benzo[a]pyrene. Therefore, CYP enzymes have been subjected to a number of rational design studies to enhance their catalytic performance. Based on the crystal structures of CYP101, selective mutations were performed on the active site residues F87 and Y96 of the enzyme [12]. For all PAH substrates studied, the absolute oxidation rates (approximately 1 min^{-1}) of the mutants, Y96A, Y96F, F87A/Y96A and F87L/Y96F, were increased by two to three orders of magnitude relative to the wild-type enzyme. In a similar study, based on the crystal structure of the enzyme, Carmichael and Wong [13] introduced two mutations into CYP102, R47L and Y51F, and found that the oxidation activity of the enzyme for phenanthrene and fluoranthene was increased by 40- and 10-fold, respectively. The double mutant was then used as a basis for further engineering of the active site. When the A264G mutation was introduced to the base mutant, NADPH turnover, PAH oxidation and coupling efficiency of the enzyme was greatly improved. The most active mutants showed more than a 200-fold increase in PAH oxidation activity compared the wild-type enzyme. Another mutation, F87A, resulted in a larger space in the substrate binding pocket of the enzyme, leading to better accommodation of larger fluoranthene and pyrene molecules in the vicinity of the haem site, and hence a more efficient PAH oxidation. In another study to engineer the CYP102 enzyme for PAH oxidation, Li et al. [14] created a triplet mutant, A74G/F87V/L188Q, with improved activity on naphthalene, fluorine, acenaphthalene, acenaphthylene and 9-methylanthracene. It was found that first mutation to F87 residue improved the enzyme activity toward PAHs by two to three orders of magnitude. The second mutation of L188G significantly increased the enzyme activities towards all three-ring PAHs by as much as 30-fold. The third substitution of A74G increased the NADPH consumption rates, and consequently the enzyme activities towards all PAHs. In total, the activities of the triplet mutant towards all the PAHs studied resulted in enzyme activities two to four orders of magnitude higher than that of the wild-type enzyme.

As can be seen from the CYP enzyme engineering studies above, NADPH consumption plays an important role in the activity of the enzymes. However, one of the main challenges facing the application of isolated CYP enzymes to bioremediation is the need to regenerate the expensive cofactor, NAD(P)H, which is consumed in the oxidation reaction. One approach to expand the practical utility of CYP is to eliminate the cofactor requirement. Directed evolution was used to create CYP101 mutants that hydroxylated naphthalene in the absence of the cofactor NAD(P)H via the ‘peroxide shunt’ pathway [15]. This process yielded several mutants with 20-fold improvements in naphthalene hydroxylation activity relative to the wild-type enzyme. Previously, it has been difficult to improve the thermostability of the P450 enzymes by protein engineering, because it is a multicomponent enzyme that depends on thermolabile cofactors. However, with the “peroxide shunt” pathway, the need of a cofactor and a reductase domain has been negated. This has allowed the thermostability of the P450 enzyme to be improved via directed evolution [16].

4. Polychlorinated biphenyls

Polychlorinated biphenyls are a class of chemicals consisting of 209 member compounds, collectively known as congeners. These compounds differ by their degree of chlorination and the position of the chlorinated sites. Since the 1930s, PCBs have had a wide range of applications from an extender in insecticide to an insulator in transformer production. Although the production and use of PCBs were phased out in many countries in the mid-1980s due to their toxicity and adverse effects on humans and wildlife, the compounds are still ubiquitous throughout the global environment and its biota because of their resistance to biodegradation [17].

PCBs can be degraded by microorganisms via a meta-cleavage pathway to yield tricarboxylic acid cycle intermediate and (chloro)benzoate (CBA). The initial step in the aerobic biodegradation of PCBs is the dioxygenation of PCB congeners by the biphenyl dioxygenase enzyme. In this step, the enzyme catalyzes the incorporation of two hydroxyl groups into the aromatic ring of a PCB congener, which increases the reactivity of the PCBs, rendering them more susceptible to enzymatic ring fission reactions [18]. Biphenyl dioxygenase is a multicomponent enzyme consisting of a terminal dioxygenase (made up of a large α and a small β subunit), ferredoxin and ferredoxin reductase encoded by the *bph* operon [19]. The substrate recognition of the enzyme is controlled by the large α subunit encoded by the *bphA* gene. Although the *bphA* gene is similar between bacteria species, the sub-

strate specificity of the biphenyl dioxygnease enzymes can differ greatly. For example, although the *Burkholderia cepacia* strain LB400 and *Pseudomonas pseudoalcaligenes* strain KF707 both show near identical sequences in their *bph* operons, the biphenyl dioxygenase enzyme from LB400 preferentially deoxygenates *ortho*-substituted PCBs, while that from KF707 preferentially deoxygenates *para*-substituted PCBs [20,21]. Earlier studies to engineer the biphenyl dioxygenases have been reviewed by Furukawa [22].

By targeting a fragment of *bphA* gene that is critical for enzyme specificity and using DNA shuffling techniques to shuffle particular gene fragments from *B. cepacia* strain LB400, *C. testosteroni* B-365 and *Rhodococcus globerulus* P6, Barriault et al. were able to obtain variants with superior degradation capabilities for PCBs [23]. The hybrid *BphA*, II-9, was able to oxygenate 2,6-dichlorobiphenyl, which is a very persistent PCB congener, by up to 58% after 18 h. Both parental *BphA* enzymes of II-9 (*B. cepacia* strain LB400 and *C. testosteroni* B-365) could only oxygenate the same PCB congener by less than 10%. II-9 also showed marked improvement in activity towards 3,3′-, 4,4′-, 2,3,4′- and 2,3,4′-congeners relative to the primary enzymes. Using a rational design approach, Suenaga et al. developed a three-dimensional model of the KF707 biphenyl dioxygenase enzyme, BphA1, in *P. pseudoalcaligenes* based on crystallographic analyses of the naphthalene dioxygenase enzyme from *Pseudomonas* sp. strain NCIB 9816-4 [24]. From the model, key positions near the active site of the enzyme were chosen for site-directed mutagenesis. The resulting mutants showed altered regiospecificities for various PCB congeners compared to the wild-type enzyme. The mutants I335F, T376N and F377L were able to degrade 2,5,2′,5′-tetrachlorobiphenyl, a PCB congener that is not degradable by the wild-type biphenyl dioxygenase.

Although the oxidative enzymes encoded by the *bph* gene operon confer upon microorganisms the ability to degrade PCBs, the metabolite from the degradation, (chloro)benzoate, cannot be further degraded by these enzymes. The plasmid pE43 contains the oxygenolytic *ortho*-dechlorination *ohb* gene, originally found in *Pseudomonas aeruginosa*, whereas the plasmid pPC3 carries the hydrolytic *para*-dechlorination *fcb* gene from *Arthrobacter globiformis*. By individually transforming these recombinant plasmids into PCB-cometabolizing *C. testosteroni* VP44, two recombinant variants, VP(pE43) and VP(pPC3), capable of using *ortho*- and *para*-chlorobiphenyls (CBs) as sole carbon sources have been obtained [25]. The parental strain, VP44, grew only on low concentrations of 2- and 4-CBs and accumulated stoichiometric amounts of the corresponding CBA, while the recombinant variants were able to grow on and dechlorinate 2- and 4-CBs by up to 95%. However, complications may arise when applied to higher chlorinated PCBs, especially those chlorinated on both aromatic rings, as no dehalogenation of chloropentadiene, which is a metabolic product of PCB chlorinated on both aromatic rings, has been documented [26]. Natural evolutionary processes may give rise to microorganisms that can dehalogenate chloropentadiene in due time, but the process may take several years or even decades. Biomolecular engineering can be applied to shorten this process of developing a novel enzyme that can carry out this reaction.

5. Pesticides

5.1. Atrazine

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-*s*-triazine) belongs to a class of *s*-triazine herbicides first introduced in the 1950s. It has since been widely used for weed control in agricultural production of crops such as maize, sorghum and sugar cane. Despite containing only one chlorine constituent, atrazine is recalcitrant to biodegradation, with a reported half-life of greater than 170 days in soils containing atrazine-degrading microorganisms [27]. Due to its recalcitrance, atrazine is frequently detected in surface and ground water samples, posing a direct risk to humans via potable water consumption.

Of the various atrazine-degrading microorganisms isolated, *Pseudomonas* sp. ADP has been most closely studied to elucidate the genes and enzymes responsible for atrazine metabolism [28–30]. In the biodegradation of atrazine, *Pseudomonas* sp. ADP converts atrazine to cyanuric acid using the AtzA, B and C enzymes. AtzA first transforms atrazine to hydroxyatrazine and AtzB then catalyzes the hydrolytic deamidation of hydroxyatrazine to yield *N*-isopropylammelide. Finally, AtzC, which is also a hydrolytic deamidase like AtzB, converts *N*-isopropylammelide to cyanuric acid. Cyanuric acid is subsequently mineralized to carbon dioxide and ammonia by enzymes that are commonly found in soil bacteria. The TriA enzyme is closely related to AtzA, but unlike AtzA it initiates the hydrolysis of *s*-triazines by deamination instead of dechlorination [31]. As microorganisms capable of metabolizing xenobiotics normally appear only a few decades after the compound is introduced to the environment, Atz and TriA are enzymes that have likely evolved to degrade atrazine only recently. Given their short history, these two enzymes are likely to be highly evolvable and directed evolution holds great prospects of accomplishing in the laboratory what nature may not have had time to accomplish in the environment, i.e. to make the enzymes highly proficient in atrazine degradation [32].

Raillard et al. applied directed evolution by DNA shuffling to the *atzA* and *triA* genes in order to explore the substrate specificities of the resulting enzymes, and to acquire a better understanding of the possible distribution of novel functions in protein sequence space [33]. Both parental enzymes and a library of 1600 variants were screened against a library of 15 triazine compounds. The shuffled library contained enzymes that hydrolyzed certain substrates that were not hydrolyzed by either parent, for example, prometon, prometryn and *N*-methylaminopropazine. For triazines for which parental enzymes showed some activity, the library yielded enzymes of

increased transformation rates by up to 150-fold. Although the degradation of atrazine was not improved greatly in this study, it was shown that there is a great richness of substrate specificities in the small region of sequence space that can be accessed by permutating the two highly homologous enzymes. Overall, this research demonstrates the great potential for improvement in the degradative abilities of AtzA and TriA via directed evolution techniques.

5.2. Organophosphates

Organophosphates (OP) are highly toxic neurotoxins used in insecticides and chemical warfare agents. Included in the organophosphate group are paraoxon, parathion, chlorpyrifos disulfoton, ruelene, carbophenothion and dimeton. The neurotoxicological properties of this class of compounds are mainly due to its ability to suppress acetylcholinesterase and as a result, prevent acetylcholinesterase from breaking down acetylcholine at the synaptic junction. These compounds have also been associated with pathology and chromosomal damage associated with bladder cancer [34].

Bacterial phosphotriesterase (PTE), also known as organophosphorus hydrolase (OPH) is a highly efficient hydrolytic enzyme that can hydrolyze a broad range of organophosphates [35]. PTE catalyzes the cleavage of P–O, P–F or P–S bonds in these organophosphates. Although PTE is thought to have only evolved within the last 50 years, its hydrolytic ability is truly remarkable. With paraoxon, its preferred substrate, it has a k_{cat} of about 2280 s^{-1} and a k_{cat}/K_M of 6.2×10^7 $M^{-1}s^{-1}$, which are very close to the maximum diffusion-controlled limit [36]. As such, application of PTE to the degradation of organophosphates has attracted considerable research interest. Although PTE can hydrolyze a variety of organophosphates, it generally prefers the S_p-enantiomers of organophosphate over the R_p-enantiomers, with the kinetic constants of the S_p-enantiomers being higher by one to two orders of magnitude [37]. To make PTE more effective for the catalytic degradation of organophosphates, the overall rate of hydrolysis for all stereoisomers must be increased. The three-dimensional structure of PTE from *Pseudomonas diminuta* with a bound substrate analogue, diethyl 4-methylbenzylphosphonate, has shown that there are three distinct hydrophobic binding pockets responsible for the orientation of substrates in the enzyme's active site [38,39]. These three binding pockets have been named the small, large and leaving group subsites.

To determine the role played by each subsite and the key amino acid residues in the reactivity and stereospecificity of PTE, Chen-Goodspeed et al. [39,40] carried out site-directed mutagenesis on key residues in the three subsites and measured the stereospecificity of the variants using a series of asymmetric organophosphates. The substitution of Ile106 by a smaller alanine residue resulted in an enlargement of the small subsite and virtually eliminated a 20–90-fold preference for S_p-enantiomers of some chiral substrates. A combined mutation of I106G/F132G, which also enlarged the small subsite, brought about a k_{cat} increase of up to 270-fold for some of the R_p-enantiomers without sacrificing the high turnover rates for the S_p-enantiomers, which is a highly desirable property for the remediation of a racemic mixture of organophosphates. When the His257 residue in the large subsite was mutated to a tyrosin residue, which reduced the size of the large subsite, the kinetic parameters of PTE on all tested S_p-enantiomers were reduced. This indicates that His257 plays an important role in the stereoselectivity of PTE [40].

Another drawback of PTE is that its effectiveness in hydrolysis varies dramatically for different organophosphate substrates. For example, some widely used insecticides such as methyl parathion, chlorpyrifos and diazinon are hydrolyzed 30–1000 times slower than paraoxon, the preferred substrate of PTE, due to unfavorable active site interactions [41]. In order to enhance the effectiveness of PTE towards poorly degraded substrates, Cho et al. adopted a directed evolution approach to enhance PTE catalytic performance [42]. The substrate chosen was methyl parathion, which is used worldwide on cotton, wheat, peach, barley and rice crops, but is poorly degraded by PTE. As organophosphates are not readily taken up by cells, screening for PTE expressed intracellularly is inadequate. To overcome this problem, a generalized selection scheme using a cell surface-displayed PTE library was developed to isolate improved variants. Following the random mutagenesis of the 1.1 kb *opd* gene fragment, which encodes PTE, the mutated *opd* fragments were subcloned into a surface-display vector and the mutant library was transformed into *Escherichia coli*. The transformed variants were then subjected to a top agar prescreening assay, based on a colorimetric reaction, where the hydrolysis of methyl parathion produces *p*-nitrophenol, a yellow colored compound. The intensity of the yellow color formed is proportional to the hydrolytic activity. Potential variants were then screened with a 96-well plate assay. This screening method can be easily extended to the directed evolution of other organophosphates such as diazinon and chlorpyrifos as these organophosphates have a common hydrolysis product, *p*-nitrophenol. Two rounds of DNA shuffling resulted in clone 22A11 that had the same level of PTE expression as the wild-type enzyme, but was 64 times more active. The purified enzyme from this clone displayed a specific activity 25 times greater than the wild-type enzyme. All improved variants selected for sequencing showed two common mutations—I274N and H257Y. It is believed that the mutation of H257 to tyrosine reduces the size of the larger binding pocket, thus favoring the binding of smaller substituents, which is the methyl group of methyl paraoxon in this case. Interestingly, this mutation was identified by Chen-Goodspeed et al. [39,40] as well, albeit using the rational design approach. As the I274N mutation is located 10 Å away from the active site, it is difficult to tell how activity is improved by this mutation, but it is clear that this important substitution is impossible to predict solely on structural information alone. The ability to identify dis-

tal mutations that involve subtle, long-range interactions is precisely what makes directed evolution such a powerful technique.

Using a novel strategy called in vitro compartmentalization (IVC), Griffiths and Tawfik managed to improve the k_{cat} of the already very efficient PTE on paraoxon [43]. This method is based on the synthesis of libraries of microbeads, each displaying a gene; the protein it encodes and the product of catalysis (Fig. 2). In this study, a gene library was created by mutagenizing the codons encoding for the

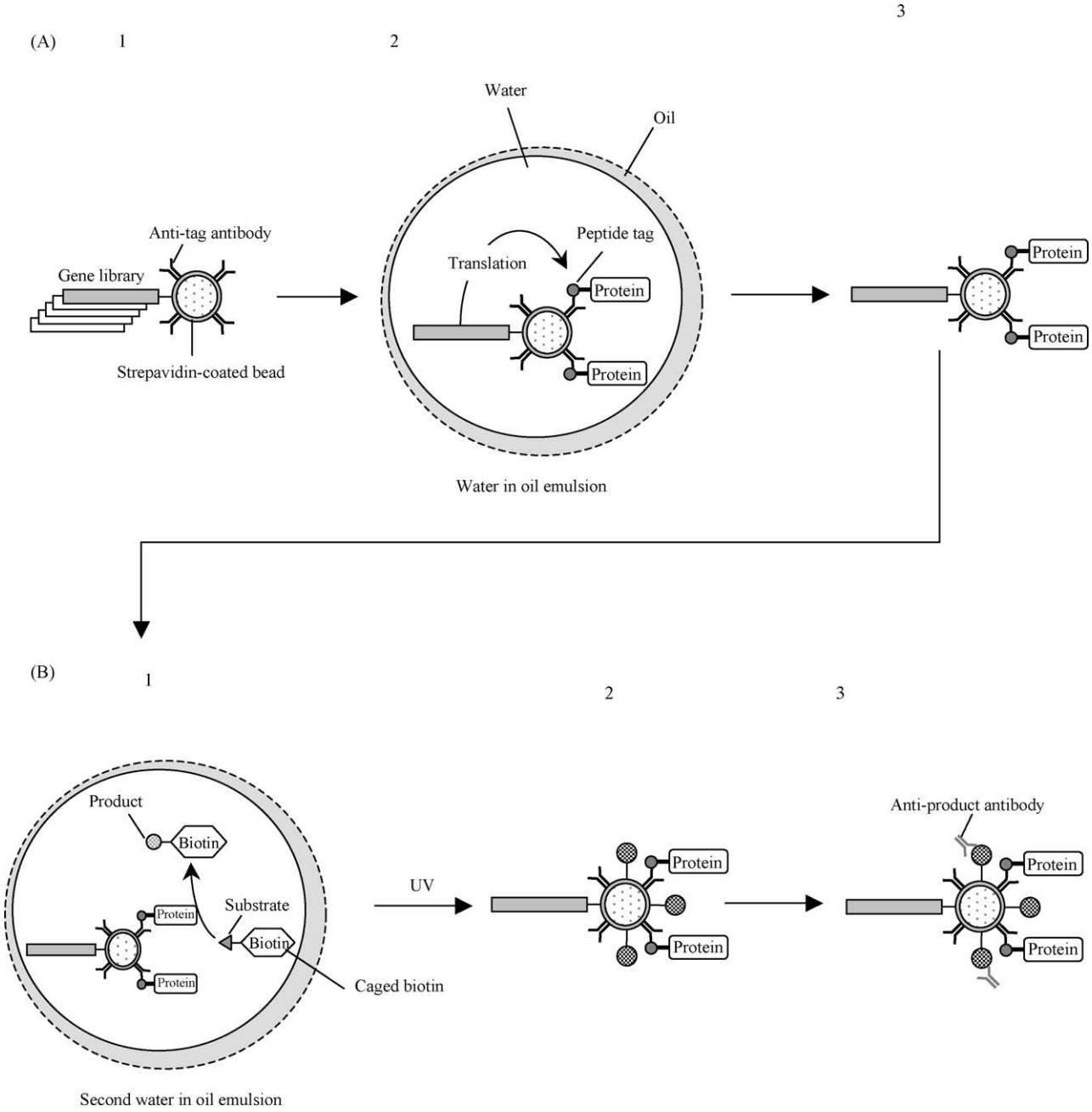

Fig. 2. Scheme for in vitro compartmentalization (IVC) selection (adapted from [43]). (A) Synthesis of microbead-display libraries. (1) A library of variant genes, each with a common epitope tag, is linked to streptavidin-coated beads carrying antibodies that bind to the epitope tag. There is less than one gene per bead on average. (2) The beads are individually compartmentalized in a water-in-oil emulsion, and then transcribed and translated in vitro. The translated proteins attach to the bead and create a linkage between the genes and the proteins they encode. (3) The emulsion is broken and the microbeads carrying the display libraries are isolated. (B) Selection of enzyme by compartmentalization. (1) Microbead-display libraries are compartmentalized in a second water-in-oil emulsion and a soluble substrate attached to caged biotin is added. Compartments with beads-displaying active enzymes will convert the substrate to product. The emulsion is then irradiated with UV to uncage the biotin. (2) Products produced in compartments with active enzymes become attached to the gene via the bead. (3) The emulsion is broken and the beads are incubated with anti-product antibodies. Beads coated by the products, gene and protein can then be enriched using affinity purification or flow cytometry, after reacting with a fluorescently labeled antibody.

residues defining the entrance to the leaving group site and the small subsite of PTE. The resulting library had an overall diversity of 3.4×10^7 variants. Libraries of proteins displayed on microbeads were first created by translation in an emulsion and were then recompartmentalized in a second emulsion to select for catalysis. Finally, fluorescently labeled anti-product antibodies were added to the microbeads and sorting of the resultant mixture was conducted using flow cytometry. In this process, clones with higher PTE activity were enriched at the expense of those with lower activity. After several rounds of enrichment, a variant which had a k_{cat} 63 times faster than the already very fast wild-type was isolated. The IVC method provided a phenotype–genotype linkage, which allows for a more effective selection of variants from a library. It also enables a simultaneous and direct selection for all enzymatic properties: substrate recognition, formation of a specific product, rate acceleration and turnover. Prior to this study, compartmentalization methods had only been used for selection of libraries in which enzymatic reaction and translation occurred in the same environment [44,45]. However, optimal conditions for translation and enzymatic reaction may not be the same, and hence the conditions selected may sacrifice optimal catalysis conditions for better translation of the proteins or vice versa. The IVC strategy overcomes this limitation by splitting the translation and enzymatic reaction steps into two different emulsions.

6. Future challenges

Biomolecular engineering can be successfully used to improve the capabilities of the enzymes or microorganisms in bioremediation systems. However, there are several limitations. Firstly, creation of enzymes with novel functions represents an overwhelming challenge in biomolecular engineering. Research is usually focused on altering enzymes that can perform a reaction similar to the desired one. Thus, it might be difficult to apply biomolecular engineering to the bioremediation of novel pollutants, which are not known to be biodegradable. Perhaps such an endeavor might be possible with rational design in the future when our knowledge of the protein structure–function, folding, mechanism and dynamics is significantly improved.

Furthermore, even if a genetically engineered microorganism (GEM) with enhanced capabilities is successfully created by biomolecular engineering, it still faces a number of significant constraints regarding its application. GEMs released into the environment may have a decreased level of fitness and may not survive due to the extra energy demands imposed by the presence of foreign genetic material in the cell. Also, unless there is a mechanism to control the spread of the mobile genetic elements (MGEs), such as recombinant plasmids containing antibiotic resistance markers, there remains a credible risk of MGEs entering the environment and being acquired by undesirable organisms. Public acceptability of releasing GEMs into the environment has led to strict regulations by government bodies such as the US Environmental Protection Agency (EPA). As a result, few microorganisms isolated have reached the stage of field application. The controlled release of *Pseudomonas fluorescens* HK44, which possesses a naphthalene catabolic plasmid (pUTK21) mutagenized by transposon insertion of *lux* (bioluminescent) genes, for biodegradation monitoring and control represents the first and only genetically engineered microorganism approved for field testing in the United States for bioremediation purposes [46]. To gain approval for field testing, recombinant *E. coli* expressing AtzA was first killed by cross-linking with glutaraldehyde before it was bioaugmented into atrazine contaminated soil [47]. This method was found to be much more efficient than the atrazine degradation achieved by indigenous microbes without supplements. Although these studies involved controlled release of GEMs, it is hoped they will provide a greater understanding of GEMs and in the process gain more public acceptance for such bioremediation techniques. However, much work still needs to be done before field applications of engineered microorganisms can be realized. The viability of GEMs for bioremediation has been discussed in greater detail in various other reviews [48–50].

7. Conclusion

Human activities have brought about widespread pollution of the natural environment. A number of organic pollutants, such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and pesticides are resistant to degradation and represent an ongoing toxicological threat to both wildlife and human beings. Over recent years, a growing number of potential hazards linked to the ubiquitous presence of POPs in the environment have been reported. Bioremediation is an attractive alternative to traditional physico-chemical techniques for the remediation of these POPs at a contaminated site, as it can be more cost-effective and it can selectively degrade the pollutants without damaging the site or its indigenous flora and fauna. However, despite being hailed as a panacea to the safe and effective solution to contaminated environmental media, bioremediation technologies, to date, have had limited applications due to the challenges of substrate and environmental variability, as well as the limited biodegradative potential and viability of naturally occurring microorganisms. In particular, the engineering and environmental release of GEMs has run into both technical and ethical obstacles, leading to severe constraints for their effective application in the field. Now, with the recent advances in biomolecular engineering, the prospect of short-circuiting the process of natural evolution to degrade environmental xenobiotic pollutants has been created. This has opened exciting new vistas for enhancing bioremediation programs in the coming years.

References

[1] United Nation Environmental Program (UNEP). Stockholm convention on persisitent organic pollutants. Stockholm, Sweden; 2001.

[2] Romantschuk M, Sarand I, Petanen T, Peltola R, Jonsson-Vihanne M, Koivula T, et al. Means to improve the effect of in situ bioremediation of contaminated soil: an overview of novel approaches. Environ Pollut 2000;107:179–85.

[3] Duran N, Esposito E. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Appl Catal B Environ 2000;28:83–99.

[4] Timmis KN, Pieper DH. Bacteria designed for bioremediation. Trends Biotechnol 1999;17:201–4.

[5] Ryu, DDY, Nam DH. Recent progress in biomolecular engineering. Biotechnol Prog 2000;16:2–16.

[6] Arnold FH. Design by directed evolution. Acc Chem Res 1998;31:125–31.

[7] Schmidt-Dannert C. Directed evolution of single proteins, metabolic pathways, and viruses. Biochemistry 2001;40:13125–36.

[8] Zhao HM, Giver L, Shao ZX, Affholter JA, Arnold FH. Molecular evolution by staggered extension process (StEP) in vitro recombination. Nat Biotechnol 1998;16:258–61.

[9] Stemmer WPC. Rapid evolution of a protein in-vitro by DNA shuffling. Nature 1994;370:389–91.

[10] Hammel KE, Kalyanaraman B, Kirk TK. Oxidation of polycyclic aromatic-hydrocarbons and dibenzo P-dioxins by phanerochaete-chrysosporium ligninase. J Biol Chem 1986;261:6948–52.

[11] Bulter T, Alcalde M, Sieber V, Meinhold P, Schlachtbauer C, Arnold FH. Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. Appl Environ Microbiol 2003;69:987–95.

[12] Harford-Cross CF, Carmichael AB, Allan FK, England PA, Rouch DA, Wong LL. Protein engineering of cytochrome P450(cam) (CYP101) for the oxidation of polycyclic aromatic hydrocarbons. Prot Eng 2000;13:121–8.

[13] Carmichael AB, Wong LL. Protein engineering of Bacillus megaterium CYP102—the oxidation of polycyclic aromatic hydrocarbons. Eur J Biochem 2001;268:3117–25.

[14] Li QS, Ogawa J, Schmid RD, Shimizu S. Engineering cytochrome P450BM-3 for oxidation of polycyclic aromatic hydrocarbons. Appl Environ Microbiol 2001;67:5735–9.

[15] Joo H, Lin ZL, Arnold FH. Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. Nature 1999;399:670–3.

[16] Salazar O, Cirino PC, Arnold FH. Thermostabilization of a cytochrome p450 peroxygenase. Chembiochem 2003;4:891–3.

[17] Alcock RE, Behnisch PA, Jones KC, Hagenmaier H. Dioxin-like PCBs in the environment-human exposure and the significance of sources. Chemosphere 1998;37:1457–72.

[18] Bruhlmann F, Chen W. Tuning biphenyl dioxygenase for extended substrate specificity. Biotechnol Bioeng 1999;63:544–51.

[19] Erickson BD, Mondello FJ. Nucleotide sequencing transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain-LB400. J Bacteriol 1992;174:2903–12.

[20] Erickson BD, Mondello FJ. Enhanced biodegradation of polychlorinated-biphenyls after site-directed mutagenesis of a biphenyl dioxygenase gene. Appl Environ Microbiol 1993;59:3858–62.

[21] Gibson DT, Cruden DL, Haddock JD, Zylstra GJ, Brand JM. Oxidation of polychlorinated-biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J Bacteriol 1993;175:4561–4.

[22] Furukawa K. Engineering dioxygenases for efficient degradation of environmental pollutants. Curr Opin Biotechnol 2000;11:244–9.

[23] Barriault D, Plante MM, Sylvestre M. Family shuffling of a targeted bphA region to engineer biphenyl dioxygenase. J Bacteriol 2002;184:3794–800.

[24] Suenaga H, Watanabe T, Sato M, Ngadiman, Furukawa K. Alteration of regiospecificity in biphenyl dioxygenase by active-site engineering. J Bacteriol 2002;184:3682–8.

[25] Hrywna Y, Tsoi TV, Maltseva OV, Quensen JF, Tiedje JM. Construction and characterization of two recombinant bacteria that grow on *ortho*- and *para*-substituted chlorobiphenyls. Appl Environ Microbiol 1999;65:2163–9.

[26] Brenner V, Arensdorf JJ, Focht DD. Genetic construction of PCB degraders. Biodegradation 1994;5:359–77.

[27] Radosevich M, Traina SJ, Tuovinen OH. Biodegradation of atrazine in surface soils and subsurface sediments collected from an agricultural research farm. Biodegradation 1996;7:137–49.

[28] de Souza ML, Wackett LP, Boundymills KL, Mandelbaum RT, Sadowsky MJ. Cloning, characterization, and expression of a gene region from *Pseudomonas* sp. strain Adp involved in the dechlorination of atrazine. Appl Environ Microbiol 1995;61:3373–8.

[29] de Souza ML, Sadowsky MJ, Wackett LP. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP: gene sequence, enzyme purification, and protein characterization. J Bacteriol 1996;178:4894–900.

[30] Sadowsky MJ, Tong ZK, de Souza M, Wackett LP. AtzC is a new member of the amidohydrolase protein superfamily and is homologous to other atrazine-metabolizing enzymes. J Bacteriol 1998;180:152–8.

[31] Seffernick JL, Shapir N, Schoeb M, Johnson G, Sadowsky MJ, Wackett LP. Enzymatic degradation of chlorodiamino-*s*-triazine. Appl Environ Microbiol 2002;68:4672–5.

[32] Wackett LP. Directed evolution of new enzymes and pathways for environmental biocatalysis. Ann Ny Acad Sci 1998;864:142–52.

[33] Raillard S, Krebber A, Chen YC, Ness JE, Bermudez E, Trinidad R, et al. Novel enzyme activities and functional plasticity revealed by recombining highly homologous enzymes. Chem Biol 2001;8:891–8.

[34] Webster LR, McKenzie GH, Moriarty HT. Organophosphate-based pesticides and genetic damage implicated in bladder cancer. Cancer Genet Cytogenet 2002;133:112–7.

[35] Hong SB, Raushel FM. Metal-substrate interactions facilitate the catalytic activity of the bacterial phosphotriesterase. Biochemistry 1996;35:10904–12.

[36] Hong SB, Raushel FM. Stereochemical preferences for chiral substrates by the bacterial phosphotriesterase. Chem Biol Interact 1999;119–120:225–34.

[37] Hong SB, Raushel FM. Stereochemical constraints on the substrate specificity of phosphotriesterase. Biochemistry 1999;38:1159–65.

[38] Vanhooke JL, Benning MM, Raushel FM, Holden HM. Three-dimensional structure of the zinc-containing phosphotriesterase with the bound substrate analog diethyl 4-methylbenzylphosphonate. Biochemistry 1996;35:6020–5.

[39] Chen-Goodspeed M, Sogorb MA, Wu FY, Hong SB, Raushel FM. Structural determinants of the substrate and stereochemical specificity of phosphotriesterase. Biochemistry 2001;40:1325–31.

[40] Chen-Goodspeed M, Sogorb MA, Wu FY, Raushel FM. Enhancement, relaxation, and reversal of the stereoselectivity for phosphotriesterase by rational evolution of active site residues. Biochemistry 2001;40:1332–9.

[41] Dumas DP, Caldwell SR, Wild JR, Raushel FM. Purification, properties of the phosphotriesterase from *Pseudomonas diminuta*. J Biol Chem 1989;264:19659–65.

[42] Cho CM, Mulchandani A, Chen W. Bacterial cell surface display of organophosphorus hydrolase for selective screening of improved hydrolysis of organophosphate nerve agents. Appl Environ Microbiol 2002;68:2026–30.

[43] Griffiths AD, Tawfik DS. Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization. EMBO J 2003;22:24–35.

[44] Tawfik DS, Griffiths AD. Man-made cell-like compartments for molecular evolution. Nat Biotechnol 1998;16:652–6.
[45] Ghadessy FJ, Ong JL, Holliger P. Directed evolution of polymerase function by compartmentalized self-replication. Proc Natl Acad Sci USA 2001;98:4552–7.
[46] Ripp S, Nivens DE, Ahn Y, Werner C, Jarrell J, Easter JP, et al. Controlled field release of a bioluminescent genetically engineered microorganism for bioremediation process monitoring and control. Environ Sci Technol 2000;34:846–53.
[47] Strong LC, McTavish H, Sadowsky MJ, Wackett LP. Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase. Environ Microbiol 2000;2:91–8.
[48] Giddings G. Tansley review no. 99—the release of genetically engineered micro-organisms and viruses into the environment. New Phytol 1998;140:173–84.
[49] Sayler GS, Ripp S. Field applications of genetically engineered microorganisms for bioremediation processes. Curr Opin Biotechnol 2000;11:286–9.
[50] Watanabe ME. Can bioremediation bounce back? Nat Biotechnol 2001;19:1111–5.