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Evolution of Efficient Pathways for Degradation of Anthropogenic Chemicals

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Abstract

Anthropogenic compounds used as pesticides, solvents, and explosives often persist in the environment and can cause toxicity to humans and wildlife. The persistence of anthropogenic compounds is due to their recent introduction into the environment; microbes in soil and water have had relatively little time to evolve efficient mechanisms for degradation of these novel compounds. Some anthropogenic compounds are easily degraded, while others are degraded very slowly or only partially, leading to accumulation of toxic products. This review examines the factors that affect the ability of microbes to degrade anthropogenic compounds and the mechanisms by which novel pathways emerge in nature. New approaches for engineering microbes with enhanced degradative abilities include assembly of pathways using enzymes from multiple organisms, directed evolution of inefficient enzymes, and genome shuffling to improve microbial fitness under the challenging conditions posed by contaminated environments.

Anthropogenic chemicals are widely used in agriculture, industry, medicine, and military operations. Examples include pesticides such as atrazine, pentachlorophenol (PCP), 1,3-dichloropropene, and DDT, explosives such as trinitrotoluene (TNT), solvents such as trichloroethylene, and dielectric fluids such as PCBs. There was little concern over the fate of anthropogenic chemicals in the environment until the late 20th century. Adverse effects on wildlife eventually focused attention on the fact that some anthropogenic chemicals persist in the environment because they are not readily degraded by microbes. However, some anthropogenic compounds, such as atrazine, are degraded remarkably quickly. Others, such as paraoxon, are efficiently detoxified, although not degraded. This review will examine how microbes assemble novel pathways for detoxification or degradation of anthropogenic compounds and how the efficiency of biodegradation can be enhanced by optimizing naturally occurring pathways and by patching together novel pathways using enzymes from different organisms.

Microbial enzymes that act upon anthropogenic compounds arise from promiscuous activities of previously existing enzymes. Promiscuous enzymes can evolve to become more effective catalysts as a result of selective pressure for detoxification of a toxic compound or use of a novel source of carbon, nitrogen, or phosphorus. Detoxification often requires only a single step. For example, PCP is toxic because it dissipates trans-membrane proton gradients, and thereby uncouples oxidative phosphorylation^{1,2}. *Phanerochaete chrysosporium* detoxifies PCP by methylating the hydroxyl group, thus eliminating its ability to transport protons across a lipid bilayer³.

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Complete degradation of anthropogenic compounds is more desirable than detoxification, but also more difficult, requiring evolution of multi-step pathways to convert the anthropogenic compound into an intermediate in a standard metabolic pathway. Pathways that mineralize anthropogenic compounds introduced in the early 20th century have been found in microbes isolated from contaminated soils. Two examples are shown in Figure 1. Atrazine is the most widely used pesticide in the world. Three steps that replace the substituents on the ring with hydroxyl groups convert atrazine into cyanuric acid, which is readily metabolized, either within the same microbe (e.g. *Pseudomonas* sp. strain ADP), or by other microbes.⁴ PCP is used primarily as a wood preservative. Conversion of PCP to the common intermediate β -ketoadipate requires several steps that remove three chlorine substituents, cleave the ring, and then remove the remaining two chlorine substituents^{5,6}. Studies of the evolutionary origin and quality of function of enzymes in such pathways enhance our understanding of how novel metabolic pathways have emerged throughout the history of life and provide guidance for efforts to improve biodegradation of recalcitrant pollutants.

Recruitment of promiscuous enzymes to serve new functions

Enzymes that act upon anthropogenic compounds have arisen from promiscuous activities of enzymes that served different roles in other pathways. Promiscuous activities occur as an adventitious consequence of the assemblage of reactive amino acids, metal ions, and cofactors at active sites. Promiscuous activities are generally inefficient - values for k_{cat} and k_{cat}/K_M are often orders of magnitude lower than those for the physiologically relevant activity of an enzyme. For example, *N*-acyl-homoserine lactone acylase from *Rhodococcus erythropolis* hydrolyzes various lactones with values of k_{cat}/K_M up to $1.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$; it hydrolyzes paraoxon, a phosphotriester insecticide, with a k_{cat}/K_M of $0.5 \text{ M}^{-1}\text{s}^{-1}$ ⁷. *E. coli* alkaline phosphatase hydrolyzes *p*-nitrophenylphosphate with a k_{cat}/K_M of $3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$; k_{cat}/K_M for the promiscuous hydrolysis of bis-*p*-nitrophenylphosphate is $0.05 \text{ M}^{-1}\text{s}^{-1}$ ⁸. However, even relatively feeble promiscuous activities often accelerate reactions substantially relative to uncatalyzed reactions⁹; hydrolysis of bis-*p*-nitrophenylphosphate by alkaline phosphatase is accelerated by 3×10^{11} ⁸. Thus, promiscuous activities provide an excellent starting place for evolution of new enzymes when a new activity becomes important for fitness or survival in a contaminated environment.

Promiscuous activities often result from substrate ambiguity, the ability to bind and convert molecules that resemble the normal substrate (see Figure 2a). However, promiscuous activities can also result in chemical transformations that are quite different from those accomplished using normal substrates (Figure 2b). In such cases, the enzyme catalyzes one or more elementary steps in the promiscuous reaction that resemble steps in the normal reaction. For example, dehydration of *o*-succinylbenzoate and racemization of *N*-acyl amino acids by *o*-succinylbenzoate synthase (Figure 2b) both require removal of a proton α to a carboxylate, followed by stabilization of the resulting enolate¹⁰. Fortuitously, *N*-acyl amino acids bind in the active site in a position that places the α proton in close proximity to the catalytic machinery (Figure 3)¹¹.

Our ability to exploit promiscuous activities for engineering of novel degradative pathways would be enhanced if we could identify the progenitors of enzymes that have been recruited to serve new functions in degradation of anthropogenic compounds. This is rarely possible. Given the enormous complexity of microbial ecosystems, there is no chance of finding the ancestor of a microbe that has evolved a new pathway in a contaminated environment and learning what has changed. Typically we must obtain clues to the function of progenitor enzymes by sequence comparisons. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP is a particularly striking case. This enzyme is 98% identical to melamine deaminase from *Pseudomonas* sp. strain NRRL B-12227¹². Both enzymes catalyze nucleophilic attack of water

on an s-triazine substrate; the substrate specificity is completely different even though the enzymes differ at only 9 positions. More often, however, sequence comparisons identify only the superfamily to which novel enzymes belong. For example, *cis*-3-chloroacrylic acid dehalogenase (*cis*-CAAD) and *trans*-3-chloroacrylic acid dehalogenase (CAAD) participate in a pathway in *Pseudomonas cichorii* 170 for degradation of the nematocide 1,3-dichloropropene¹³. These enzymes belong to the 4-oxalocrotonate tautomerase superfamily, but the low level of sequence identity between them and other members of the superfamily makes it difficult to identify the specific functions of their progenitors.

Clues to the origins of enzymes in pathways that degrade anthropogenic compounds can also come from their promiscuous activities. For example, a bacterial phosphotriesterase (PTE) that hydrolyzes the anthropogenic pesticide paraoxon¹⁴ with an astonishingly high k_{cat}/K_M of $> 4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ has a promiscuous lactonase activity¹⁵. Three homologues of PTE have robust lactonase activity with quorum-sensing lactones, as well as lower amounts of phosphotriesterase activity. These findings suggest that PTE may have evolved from an enzyme that quenches the signal from quorum-sensing lactones¹⁶.

The potential for recruitment of promiscuous enzymes to serve new functions in a novel pathway depends on several factors. First, the complement of enzymes is different in different organisms¹⁷, and, by extension, the complement of promiscuous activities should also be different. Thus, microbes may patch together different novel pathways because they have different collections of promiscuous enzymes. For example, degradation of 4-nitrotoluene occurs by different pathways in the Gram-negative bacterium *Pseudomonas* sp. Strain 4NT¹⁸ and the Gram-positive bacterium *Mycobacterium* Strain HL 4-NT-1^{19,20} (Figure 4).

A second important factor is the level of the promiscuous activity, which must reach a certain threshold in order to be physiologically significant. Levels of promiscuous activities in orthologous enzymes vary significantly due to neutral drift. For example, *o*-succinylbenzoate synthase from *Amycolatopsis* sp. has a relatively high-level *N*-acyl amino acid racemase activity (Figure 2b). The *N*-acyl amino acid racemase activities of the *E. coli* and *Bacillus subtilis* enzymes are more than four orders of magnitude lower.²¹ Variability in promiscuous activities means that some organisms will have the potential to evolve a new enzyme from a promiscuous activity, while others will not because the promiscuous activity is too weak to be useful.

Neutral drift affects the evolvability of promiscuous activities in two other ways. Evolvability is increased by neutral mutations that increase thermostability²². Mutations that change functions often destabilize proteins, so neutral mutations that enhance stability allow proteins to handle subsequent destabilizing mutations. Furthermore, the “acceptability” of mutations needed to enhance a promiscuous activity depends on the structural context; mutations that are beneficial in one context may be detrimental in another. A study of the possible trajectories for accumulation of five mutations in a β -lactamase that together increase bacterial resistance to cefotaxime by 100,000-fold²³ showed that only 18 of 120 possible trajectories were accessible – i.e. each mutational step produced an enzyme that either maintained or enhanced fitness. Such effects are likely to influence the evolvability of comparable promiscuous activities in different microbes.

A third factor that affects the potential for recruitment of promiscuous enzymes is the requirement for the enzyme to be expressed under conditions in which the promiscuous activity is needed. Constitutively expressed enzymes may be the most available for recruitment, even if there are potentially better catalysts available. Mutations that lead to constitutive expression have been shown to allow recruitment of promiscuous enzymes to serve new functions²⁴. Alternatively, enzymes may be recruited from among those that happen to be expressed under

the ambient conditions. This is the basis for “co-metabolism”, the accidental transformation of chemicals by promiscuous enzymes in a metabolic pathway in the presence of the chemical that normally induces expression of the enzymes in the pathway. Since there are various ways by which enzymes might become available for recruitment, more than one novel pathway might be patched together from promiscuous enzymes in a single bacterium. Indeed, *Pseudomonas putida* OU83 transforms 3-nitrotoluene via two routes²⁵; 70% is reduced to 3-aminotoluene, and 30% is converted to 3-nitrophenol, from which the nitro group is removed by an unidentified process.

A fourth factor that affects the recruitment of promiscuous enzymes is the need for the recruited enzyme to continue to serve its original function. Substrates for the normal and promiscuous reactions will each be competitive inhibitors of the other reaction. Since the affinity of the normal substrate for the active site is likely to be higher than that of the promiscuous substrate, even a robust promiscuous activity may be irrelevant *in vivo* and recruitment may be impossible unless gene duplication occurs and mutations in one gene copy diminish the affinity of the active site for the original substrate. Gene duplication occurs readily in microbes when enhanced gene dosage confers higher fitness, and is often observed after prolonged exposure of microbes to pesticides. For example, after growth of *Pseudomonas* sp. strain ADP on atrazine as a sole source of nitrogen for 320 generations, the evolved population degraded atrazine more quickly and carried a tandem duplication of *atzB*, which encodes the second enzyme of the atrazine catabolism pathway²⁶. Similarly, growth of *Ralstonia* sp. strain TFD41 in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) as a sole carbon source for 1000 generations resulted in amplification of *tfdA*, which encodes the first enzyme in the pathway for degradation of (2,4-D)²⁷. Similar processes could lead to duplication of a gene encoding an enzyme with a poor but critical promiscuous activity.

Recruitment of multiple enzymes into novel pathways

The need to recruit several enzymes to convert an anthropogenic compound into a metabolite in a standard pathway begs the question of whether all of the enzymes must be recruited simultaneously, or whether evolution of the pathway can proceed in a stepwise manner. The answer depends upon the nature of the initial compound and the other intermediates in the pathway. Consider the hypothetical pathway shown in Scheme 1 that converts an anthropogenic compound A into an intermediate in a common metabolic pathway (E). If the major benefit of the pathway is utilization of a novel carbon source, then flux through the pathway requires all of the enzymes. It does no good to convert A to B in the absence of enzymes that convert B to E. Likewise, it is of no benefit convert D to E in the absence of enzymes that convert A to D. However, in some cases, fitness can be enhanced by step-wise recruitment. For example, conversion of A to B will be beneficial if B is less toxic than A, or if conversion of A to B generates a useful by-product.

The likelihood of assembling multiple enzymes into a novel pathway is increased if their normal functions are linked. For example, several enzymes required for PCP degradation are encoded by a regulon induced by PCP²⁸. These enzymes were likely involved in degradation of a naturally occurring phenol and recruited *en masse*. Degradation of PCP also requires a reductive dehalogenase to remove chlorine substituents between the initial hydroxylation reaction and the ring cleavage reaction typical of phenol degradation pathways, since most enzymes that cleave hydroquinones and catechols cannot handle substrates with multiple chlorine substituents. Assembly of this multi-step pathway may have been relatively simple because several enzymes from a pre-existing pathway could have been recruited simultaneously along with a single reductive dehalogenase.

Factors that affect the efficiency of biodegradation

The efficiency of biodegradation of anthropogenic compounds varies enormously. Atrazine and 1,3-dichloropropene are degraded very efficiently. PCP is degraded slowly, but can be completely mineralized. PCBs and TNT are degraded very slowly, and partially degraded products accumulate. Many factors, including the structure and physical properties of the compound, the environmental compartments in which the compound accumulates, and the vagaries of the process of assembling degradative pathways from promiscuous enzymes account for these differences.

The availability of promiscuous enzymes with suitably high activity and evolvability is one of the most important factors affecting the efficiency of biodegradation. The remarkably efficient degradation of atrazine can be attributed to the ease of recruitment of enzymes that convert atrazine to a common metabolite (Figure 1)²⁹. The enzymes that convert atrazine to cyanuric acid arose from previously existing broad-specificity enzymes. Amidohydrolases that catalyze hydrolytic replacement of substituents on aromatic and heteroaromatic rings are widely distributed in nature. Active sites in this superfamily are located at the mouth of an α/β barrel and contain one or two metal ions that activate water for attack on a range of substrates, including amino acids, nucleic acids, sugars, and organophosphate esters³⁰. Adaptation of the active site to accommodate a novel substrate is accomplished by point mutations and indels in loops surrounding the active site. Evolution of more efficient enzymes for atrazine degradation would have been fostered by the growth advantage provided by release of ammonia from the substituents and s-triazine ring, and of carbon from the substituents. (The ring carbon atoms in atrazine are released as CO₂, so the s-triazine itself does not serve as a carbon source.)

Evolution of efficient pathways is difficult in cases in which a toxic intermediate is produced. PCP degradation is strikingly inefficient, probably for this reason. The poor catalytic abilities of PCP hydroxylase limit flux through the degradative pathway³¹. PCP hydroxylase is a member of a family of flavin monooxygenases that initiate degradation of phenols, which are widely occurring natural products. Enzymes in this family hydroxylate a wide range of substrates, so there should be many enzymes in soil microorganisms that could be recruited to hydroxylate PCP. However, two aspects of the reaction catalyzed by PCP hydroxylase make this step problematic. First, enzymes in this family become “uncoupled” during turnover of poor substrates when the C4a-hydroperoxyflavin intermediate fails to hydroxylate the substrate and instead eliminates H₂O₂ (Figure 5)^{32,33,34}. This process wastes NADPH and generates a toxic species; thus, recruitment of a flavin monooxygenase to hydroxylate a poor substrate inevitably leads to some toxicity. The problem is exacerbated when the substrate is hydroxylated at a position that carries a chlorine. In such cases, the initial product eliminates HCl and generates an electrophilic benzoquinone³⁵. Tetrachlorobenzoquinone is highly toxic to *E. coli* protoplasts at levels as low as 0.5 μ M³⁶. The reason that *S. chlorophenolicum* has not evolved a better version of PCP hydroxylase may be that the toxic effects of H₂O₂ and TCBQ, as well as the unproductive depletion of NADPH, overwhelm cells in which better enzymes arise.

Evolution of efficient degradative pathways is also likely to be slow when degradation is accomplished by co-metabolism by a consortium of microbes. For example, several microbes catalyze transformations of TNT via co-metabolic processes that reduce the nitro substituents, or less commonly, remove a nitro substituent. However, microbes carry out enough reactions to derive a benefit from its degradation. Evolution of an efficient degradative pathway may be more rapid when several consecutive reactions, or even the entire pathway, are found within a single microbe that can reap the advantages of accessing a novel source of carbon, nitrogen, or phosphorous.

Biodegradation can be difficult when various reactions required for mineralization occur in different environmental compartments. This is the case for PCBs. Commercial preparations of PCBs contained complex mixtures of compounds with variable numbers of chlorine substituents. Each of the 209 possible congeners presents a different challenge for biodegradation. No naturally occurring microbe is known to mineralize a PCB. Removal of chlorine substituents occurs slowly in anoxic sediments via reductive dehalogenation reactions, which are due to use of PCBs as terminal electron acceptors³⁷. This process is most efficient when several chlorine substituents are present. Thus, PCBs in sediments are converted to mixtures of less highly chlorinated congeners, but further degradation does not occur. Once most of the chlorine substituents are removed, aerobic organisms can attack the biphenyl rings using O₂-dependent dioxygenases. However, PCBs in anoxic sediments are not accessible to the aerobic microbes that could initiate breakdown of the biphenyl ring structure³⁸.

Strategies for improvement of existing pathways

The efficiency of naturally occurring pathways for degradation of anthropogenic compounds can be improved by various methods. Directed evolution can be used to improve the poor catalytic performance of enzymes. This process involves generation of a large library of variant enzymes using mutagenic PCR, DNA shuffling, or variants of these techniques (reviewed in 39), followed by identification of improved variants by selection or screening. Enzymes evolved in this manner are usually not as efficient as naturally occurring enzymes, simply because the method does not allow the extensive exploration of sequence space that occurs in vast microbial populations over geological time scales. Nevertheless, an improvement in catalytic efficiency of 10- or 100-fold could significantly enhance the efficiency of a degradative pathway.

The efficiency of degradation of anthropogenic compounds can also be limited by the toxicity of metabolic intermediates. Toxicity can result from obvious mechanisms such as covalent modification of cellular macromolecules or generation of reactive oxygen species. Toxicity can also occur due to adventitious interactions between metabolites and cellular proteins. Such problems have presumably been minimized for naturally occurring metabolites by mutations that eliminate promiscuous binding, but natural selection has had little opportunity to mitigate the effects of novel metabolites generated by degradation of anthropogenic compounds. Both problems are at least partially amenable to technological fixes.

Many naturally occurring metabolic pathways involve problematic intermediates that are toxic, unstable, or volatile. Such intermediates are typically delivered directly from the active site at which they are generated to the active site at which they are consumed, either by tunnels⁴⁰ (e.g. the amidotransferases in which ammonia formed by hydrolysis of glutamine is transferred via a tunnel the next active site to prevent loss by volatilization) or by formation of complexes between two enzymes (e.g. the proposed association that may allow unstable glutamyl phosphate to transfer from the active site of glutamyl kinase to the active site of glutamyl phosphate reductase⁴¹). Engineering of such sophisticated solutions is beyond our current abilities. However, the impact of toxic intermediates might be reduced by tuning the levels of expression of enzymes that make toxic intermediates and enzymes that consumes them. Recently developed methods hold promise for identification of the ideal levels of expression of enzymes in a pathway⁴². Synthetic promoter libraries containing variant promoters that lead to a range of expression levels have been developed for several microbes. Assessment of the effects of different combinations of expression levels could be done by installing different promoters upstream of each gene in a pathway, followed by combinatorial reassembly of the pathway and evaluation of the phenotypic consequences. An alternative strategy is suggested by the common occurrence of bifunctional enzymes that catalyze successive steps in metabolic pathways. These enzymes may have evolved because a high local concentration of the

intermediate in the vicinity of the first enzyme enhances the rate of the next step. Fusion of genes encoding enzymes that generate and use a toxic intermediate might allow more efficient conversion of a toxic intermediate before it can damage other macromolecules.

Alleviation of toxicity caused by promiscuous binding to macromolecules requires different strategies because it is impossible to anticipate what the targets might be. An approach to this problem that identifies genes for which overexpression mitigates toxicity, presumably by overcoming the inhibition of function due to promiscuous binding, is SCALES (multiscale analysis of library enrichment)⁴³. SCALES is performed by introducing libraries containing genomic DNA inserts of variable size into bacteria and growing the cells under selective conditions. Inserts that result in increased growth rate can be identified by microarray analysis of the population that remains at the end of the selection. Once target proteins are identified, directed evolution approaches can be used to evolve versions that no longer bind the toxic intermediate. This approach is powerful, but limited to strains for which microarrays, high-copy expression vectors, and efficient transformation methods are available.

Genome shuffling is another unbiased method for mitigating the effects of toxic metabolites, as well as improving fitness by other unanticipated mechanisms. Genome shuffling involves generation of mutants that have an improved phenotype, followed by multiple rounds of protoplast fusion to allow recombination between genomes^{44,45}. Genome shuffling is more rapid than standard approaches to strain improvement because recombination allows faster accumulation of beneficial mutations, as well as opportunities to eliminate deleterious mutations. Genome shuffling has been used to improve degradation of PCP by *S. chlorophenolicum*.⁴⁶ After three rounds of shuffling, several strains were obtained that degraded PCP faster and tolerated higher levels of PCP than the wild type strain. Various combinations of mutations leading to enhanced growth rate, constitutive expression of PCP degradation genes, and enhanced resistance to the toxicity of PCP and its metabolites contributed to the improved phenotypes. A disadvantage of this approach is that mechanisms responsible for strain improvement are not revealed. However, as the cost of genome sequencing and re-sequencing drops, it will become possible to identify mutations that cause improved phenotypes and to use that knowledge for rational engineering of improved strains.

Strategies for engineering novel pathways

The explosion of whole genome sequence information, advances in methods for directed evolution and high-throughput screening, and the accumulated wisdom from decades of studies of naturally occurring metabolic pathways have set the stage for efforts to design novel pathways for biodegradation of anthropogenic compounds. This approach can exploit enzymes obtained from multiple organisms and allow assembly of novel pathways in microbes that are easy to grow, amenable to genetic manipulation, and, for in situ bioremediation applications, able to survive in natural environments. Designed pathways could avoid many of the problems that limit the efficiency of biodegradation, such as the intermediacy of toxic metabolites and the need for both reductive and oxidative transformations that are often not found in microbes in a single environmental compartment.

A successful implementation of this strategy generated a strain of *Pseudomonas putida* that degrades paraoxon, a synthetic phosphotriester insecticide. No single microbe is known to completely mineralize paraoxon. Keasling and co-workers patched together enzymes from four organisms to construct a strain of *P. putida* that uses paraoxon as a sole source of carbon and phosphorus (Figure 6)⁴⁷. This strain may be useful for destroying stockpiles of insecticides and toxic nerve agents such as soman and sarin, as well as for decontaminating equipment used in insecticide application. Other examples of this approach include the engineering of

Sphingobium chlorophenolicum ATCC 39723 to degrade hexachlorobenzene⁴⁸ and of *Pseudomonas* strains to degrade 2-chlorotoluene⁴⁹.

Designing a novel pathway can become daunting when even a modest number of transformations is required. The size of “pathway space” presents both challenges and opportunities, of course. The best choice among a set of possible pathways will depend upon factors such as the thermodynamic stability of intermediates and the availability of enzymes to catalyze the necessary steps, but other factors, such as avoidance of toxic intermediates, can be important, as well.

A number of algorithms have been developed to facilitate de novo design of novel pathways. Some⁵⁰ are primarily intended for prediction of pathways for biosynthesis of valuable end-products based on criteria such as product yield and growth substrate, but others^{51,52,53} are useful for prediction of degradative pathways, as well. Some algorithms generate pathways using known reactions drawn from metabolic pathway databases such as MetaCyc⁵⁴ and KEGG⁵⁵. Others generate pathways based upon known transformations of functional groups regardless of whether an enzyme is known to catalyze a specific reaction; an example is the Pathway Prediction System associated with the University of Minnesota Biodegradation Database⁵⁶, which uses 257 biotransformation rules for 50 functional groups learned from 183 pathways for pollutant degradation.

The repertoire of enzymes for construction of novel pathways is increased by the availability of genomic resources that allow protein engineers to identify orthologous enzymes from different microbes and choose those that are most suitable in terms of flux, stability, or regulatory properties, for a particular application. Additional enzymatic capabilities are likely to be discovered as we make progress in identifying the roles of the approximately 50% of proteins of unknown function in microbial genomes. Further, uncultured microbes contribute to biodegradation of anthropogenic compounds and are thus likely to contain useful enzymes that are yet to be discovered.

Although designed pathways can most easily be constructed using enzymes that efficiently catalyze the necessary reactions, they need not be limited to steps for which enzymes are known. If an enzyme needed to catalyze a desired reaction is not known, a promiscuous enzyme can be used, particularly if directed evolution methods can improve its catalytic efficiency. A designed pathway for degradation of *cis*-dichloroethylene that utilizes two promiscuous enzymes whose activities were enhanced by directed evolution or site-saturation mutagenesis is shown in Figure 7⁵⁷. Chlorinated ethenes such as trichloroethylene and *cis*-1,2-dichloroethylene are widely used as solvents and de-greasing agents. Anaerobic degradation results in accumulation of vinyl chloride, a carcinogen; aerobic degradation generates toxic chlorinated epoxyethanes. The first step in this engineered pathway is catalyzed by a variant of toluene *o*-monooxygenase generated by DNA shuffling that has enhanced activity with *cis*-dichloroethylene⁵⁸. The second step is catalyzed by an engineered epoxide hydrolase from *Agrobacterium radiobacter* AD1 that accepts *cis*-1,2-dichloroepoxyethane as a substrate. (It is not clear whether the final steps that liberate two chloride ions and form glyoxal are non-enzymatic or catalyzed by enzymes.)

Challenges for Future Work

Challenges remain for the design of novel pathways for degradation of recalcitrant pollutants. We currently do not have a catalogue of potentially useful promiscuous activities, or even a very good idea of the types of promiscuous activities available among naturally occurring enzymes. Predictions of enzymes that might have suitable promiscuous activities can be made from searches for enzymes that catalyze similar transformations of functional groups, or for enzyme superfamilies that catalyze one or more of the elementary chemical steps required for

the desired transformation using the Structure-Function Linkage Database⁵⁹. An alternative strategy would be to use docking algorithms to identify enzymes that might bind a target molecule in the active site. Enzymes that are predicted to bind substrates in proximity to catalytic groups that would be required for the desired transformation could be screened for their ability to catalyze the desired reaction.

A second challenge is the identification of enzymes with improved activity in libraries generated by directed evolution methods. Identification of improved enzymes is straightforward when enzyme efficiency can be directly related to a selectable trait such as survival or growth rate, but more problematic when a screen for improved activity is necessary. Screening is not difficult when the reaction generates a chromophore, a pH change⁶⁰, or a fluorescence signal⁶¹. However, this is often not the case for metabolic enzymes that convert one small metabolite into another and may not tolerate substrate analogues that incorporate bulky groups needed for signal generation. Automated methods that allow assays using microliter volumes in 1536-well plates increase the size of libraries that can be interrogated⁶². New approaches that miniaturize assays to the nanoliter scale on glass slides can save reagent costs if a sensitive method of detection is available⁶³. However, these methods still require preparation of lysates from thousands of individual strains. A step toward solving this problem is the development of a “chemical complementation” method that couples formation of a reaction product with expression of a gene required for growth. Cornish and co-workers have designed a reporter system in which a small molecule is used to bridge a DNA-binding domain-receptor fusion protein and an activation domain-receptor fusion protein, resulting in activation of *lacZ* expression in yeast^{64,65}. Enzymatic cleavage of the small molecule abrogates its ability to bring together the DNA-binding and activation domains. The resulting loss of LacZ (β -galactosidase) can be detected using blue-white screening on plates containing the indicator substrate X-Gal. (Colonies that lack an enzyme that cleaves the substrate express LacZ and are blue; colonies that express an enzyme that cleaves the substrate lack LacZ and are white.) A similar system detects enzymes that form a dimeric small molecule from two substrates⁶⁶. A more general approach could build upon the work of Topp and Gallivan, who used directed evolution to generate a riboswitch that controls expression of a gene required for motility in *E. coli* in response to a particular ligand.⁶⁷ In the presence of the ligand, the motility of *E. coli* is increased. Evolution of this riboswitch to recognize the product formed by an enzyme would enable a reporter system in which the most efficient enzyme variants in a library would lead to the highest motility on plates in the presence of the substrate.

While new technologies and genomic resources have improved our abilities to engineer efficient pathways for degradation of anthropogenic compounds, practical challenges remain. Applications of engineered microbes for waste treatment are certainly within reach, but the potential of bioaugmentation - the addition of contaminant-degrading microbes - for bioremediation of contaminated soils and aquifers is controversial^{68,69}. In most cases, engineered microbes do not survive well in natural environments, which are often characterized by fluctuating conditions and intense competition from indigenous microbes. Bioaugmentation is most likely to succeed when strains that are capable of survival under particular conditions are used. This could be accomplished by transferring genes encoding an engineered pathway into a strain that is highly abundant in a contaminated environment. An alternative is to use introduced strains as vehicles for horizontal gene transfer of degradative genes to indigenous microbes. In either case, hurdles originating from heterologous expression of degradation genes will be encountered. Thus, assembly of efficient metabolic pathways in laboratory strains is only the first step toward development of effective bioremediation strategies.

Summary

Efforts to elucidate pathways for biodegradation of anthropogenic compounds and to use microbes for bioremediation began in the late 20th century with enrichment and characterization of microbes capable of degrading pollutants from contaminated sites. In some cases, strains capable of mineralizing a pollutant could be isolated, but in other cases, degradation – often only partial – required finicky consortia. These early efforts were limited to studying degradation pathways in microbes that could be cultivated, or to the frustrating task of identifying metabolites that accumulated during degradation with no way of assigning particular processes to specific microbes. Optimization was often limited to efforts to enhance growth of contaminant-degrading microbes, as genetic manipulation of relatively exotic soil microbes was difficult. A confluence of new technologies now offers entirely new ways of approaching the challenges of bioremediation. Whole genome sequencing can facilitate identification of enzymes involved in degradative pathways and allow identification of homologs in other microbes. Transcriptional profiling can reveal physiological changes that allow microbes to survive in contaminated environments. Microbial community profiling and metagenomics approaches offer the potential for discovery of degradative enzymes in uncultured microbes from contaminated environments. Novel pathways that avoid the limitations of naturally occurring pathways can be designed using computational approaches, and can incorporate reactions not found in nature by using catalytically promiscuous enzymes whose activities have been improved by directed evolution. The fitness of microbes that express degradative enzymes, either in natural or engineered pathways, can be enhanced by unbiased approaches to generate strains that have improved resistance to the toxicity of pollutants and their metabolites and the ability to survive in challenging environments. These approaches should enable development of strains that efficiently degrade recalcitrant anthropogenic compounds in diverse contaminated environments and to thereby provide environmentally benign and relatively low-cost methods for clean-up of contaminated sites and treatment of industrial wastes.

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Figure 1.

Pathways for a) degradation of atrazine in *Pseudomonas* sp. strain ADP⁴ and b) degradation of pentachlorophenol in *Sphingobium chlorophenolicum* sp. Strain ATCC 39723^{5,6}. Highlighted in blue is the part of each pathway that converts the anthropogenic compound into an intermediate in a standard metabolic pathway.

Figure 2.

Examples of promiscuous activities that a) resemble the normal transformation and are due to substrate ambiguity; and b) result in transformations that are quite different from the normal transformation, yet involve common elementary chemical steps. Values for $k_{\text{cat}}/K_{\text{M}}$ in units of $\text{M}^{-1}\text{s}^{-1}$ are shown in red for normal activities and in blue for promiscuous activities. Kinetic parameters are from references 70 (homoserine kinase), 71 (alkaline phosphatase), 10 (*o*-succinylbenzoate synthase), and 72 (tetrachlorohydroquinone dehalogenase). (*) A value for $k_{\text{cat}}/K_{\text{M}}$ for tetrachlorohydroquinone dehalogenase cannot be determined due to severe substrate inhibition⁷³.

Figure 3. Binding of a) *o*-succinylbenzoate and b) a promiscuous substrate (N-acetyl methionine) to the active site of *o*-succinylbenzoate synthase¹¹. Reprinted from Reference 11 with permission from Elsevier.

Figure 4.
Pathways for degradation of 4-nitrotoluene in *Pseudomonas* sp. Strain 4-NT and *Mycobacterium* Strain HL 4-NT-1.

Figure 5.

The consequences of recruiting a flavin monooxygenase to hydroxylate PCP include uncoupling of formation of C4a-hydroperoxyflavin and hydroxylation of substrate, which wastes NADPH and generates H_2O_2 , and formation of toxic tetrachlorobenzoquinone.

Figure 6.
An engineered pathway for degradation of paraoxon that utilizes enzymes from four different microbes⁴⁷.

Figure 7.

An engineered pathway for degradation of *cis*-1,2-dichloroethylene that uses enzymes with promiscuous activities that have been enhanced by DNA shuffling (toluene *o*-monooxygenase (TOM) from) or site-saturation mutagenesis of active site residues (epoxide hydrolase from *Agrobacterium radiobacteri* AD1 (EchA))⁵⁷.