Cleaning up our own backyard: developing new catabolic pathways to degrade pollutants

Microbial-based strategies for pollution control require metabolic pathways by which man-made compounds may be degraded. Recombination-based mutagenesis and selection procedures may be able to mimic the evolution of catabolic pathways and generate enzymes with novel specificities.

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In the past century or two, industrial manufacturing has generated large quantities of new organic chemicals, ranging in function from paints and plastics to explosives and biocides. The eventual fates of these man-made compounds (xenobiotics) was of little concern while their quantities were relatively small. As economies and industries have grown, however, the spread of xenobiotics through the environment — whether as a result of their intended use, accidental release or waste-disposal policies — has made it increasingly clear that neither the atmosphere nor the oceans have unlimited dilution capacities.

One consequence of the appearance of new organic molecules has been the remarkably rapid evolution of enzymes with new catabolic activities. Microorganisms with these new enzymes can transform xenobiotics into substrates for metabolism, thereby gaining access to a nutrient source for which there is no competition [1]. Xenobiotics may also be toxic to microorganisms, providing selective pressure to develop enzymes that can metabolize them to less toxic compounds. Unfortunately not all xenobiotics have applied sufficient selective pressure to make the evolution of degradative enzymes profitable to the microorganisms. The resulting accumulation of recalcitrant compounds through the food chain has been catastrophic for organisms at the top. Common examples are the insecticide dichlorodiphenyltrichloroethane (DDT), and polychlorinated biphenyls (PCBs), which were used to manufacture a variety of products including electrical transformers and fire retardants.

Where do new enzymes come from?

The deliberate use of microorganisms to degrade man-made pollutants (bioremediation) is an emerging technology which often has cost and feasibility advantages over traditional methods such as incineration or long term storage [2]. One of the factors determining the future of bioremediation will be our ability to find or generate microorganisms that can transform waste compounds that are not currently biodegradable. Consideration of the evolution of xenobiotic-metabolizing pathways may suggest strategies for manipulating microorganisms to develop novel catabolic enzymes.

The most likely progenitors for enzymes involved in the conversion of man-made compounds to useable metabolites are enzymes that act on naturally occurring structural analogs of the xenobiotics in question. Some need little or no modification. For example, the lignin-olytic enzymes of white rot fungi, which evolved to digest wood, can also catabolize some polycyclic aromatic hydrocarbons (PAHs; these carcinogenic compounds are ingredients in creosote as well as contaminants at manufactured-gas plants) [3,4]. Other enzymes have evolved to thermodynamic perfection in a few decades; the *Pseudomonas diminuta* phosphotriesterase is diffusion-limited in its hydrolysis of synthetic phosphotriesters such as the insecticide paraoxon, which was first made in 1950 [5].

Rational enzyme redesign

A large number of similar enzyme pathways catalyze the aerobic metabolism of various aromatic hydrocarbons. Comparisons between these pathways have provided information allowing successful modifications of their substrate specificities. The first steps in aerobic metabolism of aromatic hydrocarbons are often a pair of dioxygenation reactions. Figure 1 shows these reactions on a generic substituted aromatic ring. Following an initial dioxygenation reaction, the aromatic ring is opened by a second dioxygenase that attacks either between the two hydroxyl groups produced by the first dioxygenase (the *ortho* cleavage pathway, Fig. 1a) or adjacent to them (the *meta* cleavage pathway, Fig. 1b). A principal determinant of substrate specificity in different aromatic hydrocarbon oxidation pathways is the enzyme catalyzing the first dioxygenation [6,7].

One group of compounds oxidized in this way are PCBs, a family of 209 similar congeners (biphenyl molecules substituted with chlorines at different positions). Biphenyl dioxygenases from different pseudomonads oxidize different subsets of PCBs. Although catabolic diversity in nature is generated by random mutation, increasingly powerful computers enable us to attempt to modify enzyme activities in predictable ways. Primary sequence analyses of two different biphenyl dioxygenases showed a few small regions of divergence in one of the subunits. Site-directed mutagenesis of a region in one dioxygenase

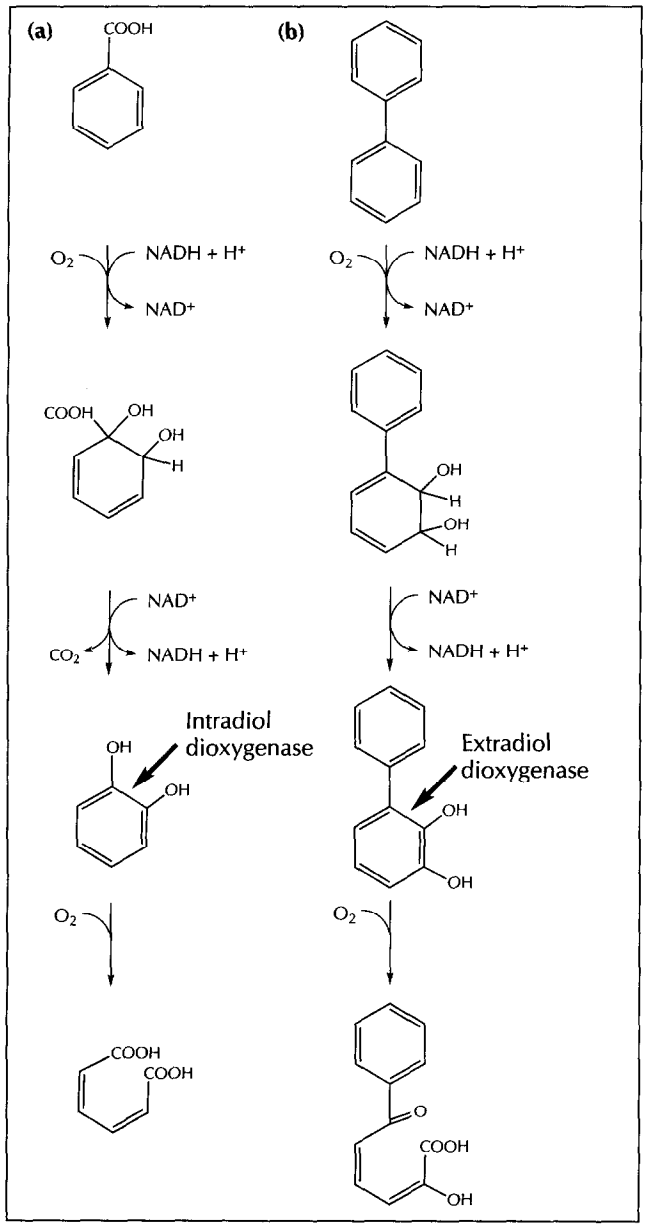

Fig. 1. Aromatic ring cleavage by sequential dioxygenation reactions. An initial dioxygenation followed by dehydrogenation converts the ring into a catechol. The catechol is then further dioxygenated and cleaved. **(a)** Benzoate and its derivatives are metabolized by the *ortho*-cleavage pathway, which uses an intradiol dioxygenase. **(b)** Biphenyl, by contrast, is oxidized by an extradiol dioxygenase in the *meta*-cleavage pathway, as is toluene [28].

to the sequence found in the other protein produced an enzyme that can oxidize a range of PCBs intermediate between those of the two original enzymes [6].

Amino acid sequence comparisons are a relatively crude tool for determining the parts of an enzyme that are important for its substrate specificity. Another widely used approach is to generate molecular dynamics simulations of enzyme X-ray crystal structures [8]. This allows the structure of an enzyme's active site to be modeled, so that predictions can be made about the effect of amino-acid substitutions in or near the active site. It should thus be possible to modify the shape of the enzyme to accommodate different substrates.

A problem that all rational approaches to enzyme design share is the extreme limitation of our current understanding of the way that amino acid sequence determines a protein's structure. The sequence space that can be explored in this way is thus confined to enzymes whose structures are known accurately, and to amino acid changes whose effect on that structure can be reasonably predicted (Fig. 2). This is only a small subset of the total sequence space available to proteins, which may be accessible (either in the past or in the future) via evolutionary mechanisms of mutation and selection.

Mutation and selection as a tool for enzyme alteration

Most randomly chosen polypeptide sequences do not fold into stable or functional proteins [9]. Nevertheless, evolution has very successfully colonized functional sequence space by trial and error. In addition to causing mutations in an enzyme's active site, 'errors' in DNA replication ensure that organisms test the effects of amino acid changes throughout the length of the protein. In some cases it has been possible to obtain mutant enzymes that can degrade compounds similar to their natural substrates simply by selecting for spontaneous mutations that allow mutant bacteria to grow on the new substrate [10]. Not only is this method considerably easier than determining an X-ray structure and finding the money for some really big computers, it can also test the effects of amino-acid changes, insertions and deletions in parts of the protein that do not have predictable roles in the structure of the active site [10,11].

Of course if the biodegradation of all xenobiotics were as simple as waiting for evolution to deal with them, pollution sites could simply be fenced off while microbes evolved to clean them up. In fact, microbes have evolved that can degrade a number of compounds including small PAHs and lightly chlorinated PCBs. For other xenobiotics, however, concentrations of compounds that are harmful to humans may not always be high enough to make them either attractive as nutrients or microbiologically toxic enough to provide a strong selective pressure for their catabolism [12]. In addition, mutations required to generate new catabolic enzymes are often not just point mutations but may be the result of exchange of genetic information between different bacteria. A combination of low densities of soil bacteria and non-selective levels of xenobiotics can thus make evolution a (relatively) slow process.

These limitations can be overcome, to a large extent, in the laboratory. In the simplest experiments, undefined soil samples from contaminated sites are incubated for prolonged periods in a chemostat (a reaction vessel in which microorganisms are incubated under regulated conditions) with the xenobiotic target, and evolution is allowed to take its course. In more directed experiments, the gene of interest is cloned and subjected to mutagenesis [13].

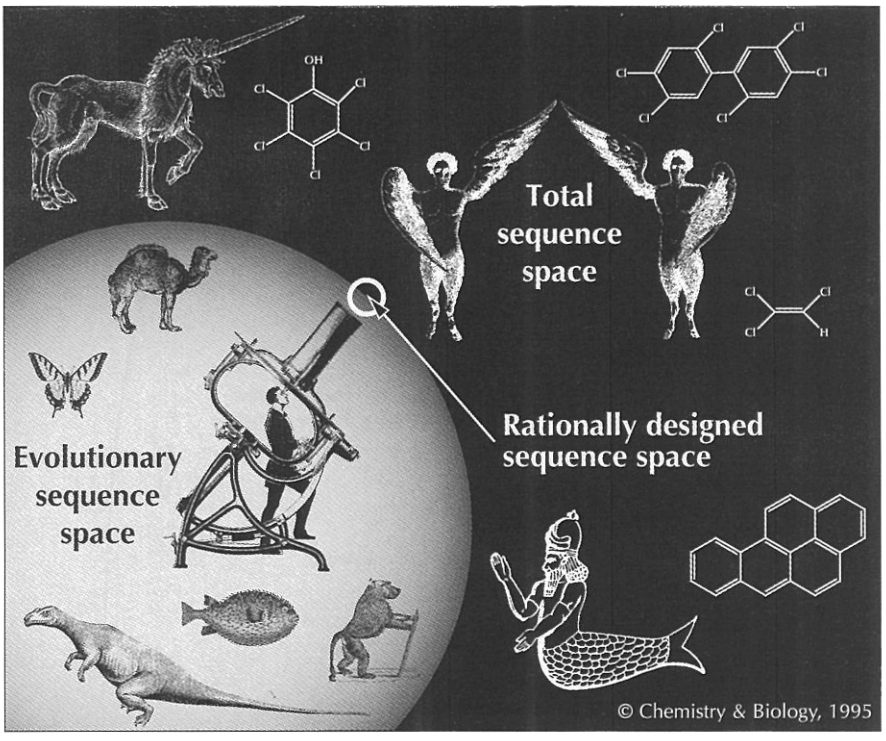

Fig. 2. Computational, evolutionary and total sequence spaces. A representation of the extent to which sequence space may be searched by structure-based methods compared with the diversity of sequence space that is available by the evolutionary mechanisms of random mutation and selection, either in the past (evolutionary sequence space) or the future (total sequence space). The structures of several compounds that are currently non-biodegradable are shown in total sequence space.

This may be done randomly throughout the gene [14,15] or by the incorporation of random sequences at defined regions of interest [16,17]. Microbes containing mutant enzymes are then either selected by their ability to resist a toxic chemical [16], use a nutrient not available to the wild type [14,17] or chosen on the basis of a screen [15]. These methods give up to a ten-fold improvement in enzyme activity toward the new substrate. A protocol that relies entirely on random mutagenesis is limited because any amino acid change is far more likely to disrupt the function of an enzyme than to improve it. Thus only a small percentage of molecules will be generated that have a single advantageous mutation and no deleterious ones. The chances of obtaining two beneficial mutations and no harmful ones become vanishingly small.

Directing evolution to explore sequence space

Evolution does not rely only on point mutations to generate diversity. Genetic recombination between phenotypically different organisms is extremely important in allowing rapid and efficient searches of sequence space [18]. Thus a better method of mutagenesis is one that not only causes point mutations, but also facilitates exchange of information between different mutants.

One such method that has been developed recently is DNA shuffling [19]. The basic protocol is shown in Figure 3. The gene of interest is mutated and then fragmented randomly with DNase. Different mutations are then allowed to recombine with each other by placing all the fragments in a polymerase chain reaction (PCR) without added oligonucleotide primers. DNA synthesis is primed off the annealed ends of overlapping fragments from the original gene, with the consequence that mutations formerly on different molecules are combined. The gene is reassembled and transformed into bacteria which are then selected for the desired phenotype. The best 100–1000 of these mutants are harvested, and their mutant plasmids isolated, pooled and subjected to a further round of fragmentation, reassembly and selection. This method has the clear advantage that it does not depend upon two advantageous and no deleterious mutations being simultaneously formed on the same plasmid molecule. Rather it allows the combination of synergistic advantageous mutations, which can then be selected. The resistance of *Escherichia coli* to the antibiotic cefotaximine was improved 1000-fold more by this recombinogenic procedure than by a simple error-prone PCR mutagenesis [19].

With DNA shuffling several properties of an enzyme can be changed simultaneously. For example, the regulation of an enzyme's expression by substrates or substrate analogs will be subjected to mutagenic and selective pressures at the same time that its structural elements are altered. Evolution-based methods depend solely on phenotypic selection, rather than on educated guesswork at how best to alter an enzyme's coding sequence. Beneficial changes can thus be obtained without having to determine precise details of the structure, mechanism and regulation of the enzyme.

Mixing and matching enzyme parts

The bacterial catabolic pathways responsible for aerobic degradation of aromatic and chlorinated aromatic hydrocarbons are encoded by groups of genes carried on large plasmids [20–22]. These plasmids appear to have arisen by horizontal gene transfer, recombination and transposition allowing the recruitment of enzymes or groups of enzymes from different microorganisms [21]. There is a good deal of similarity between the different

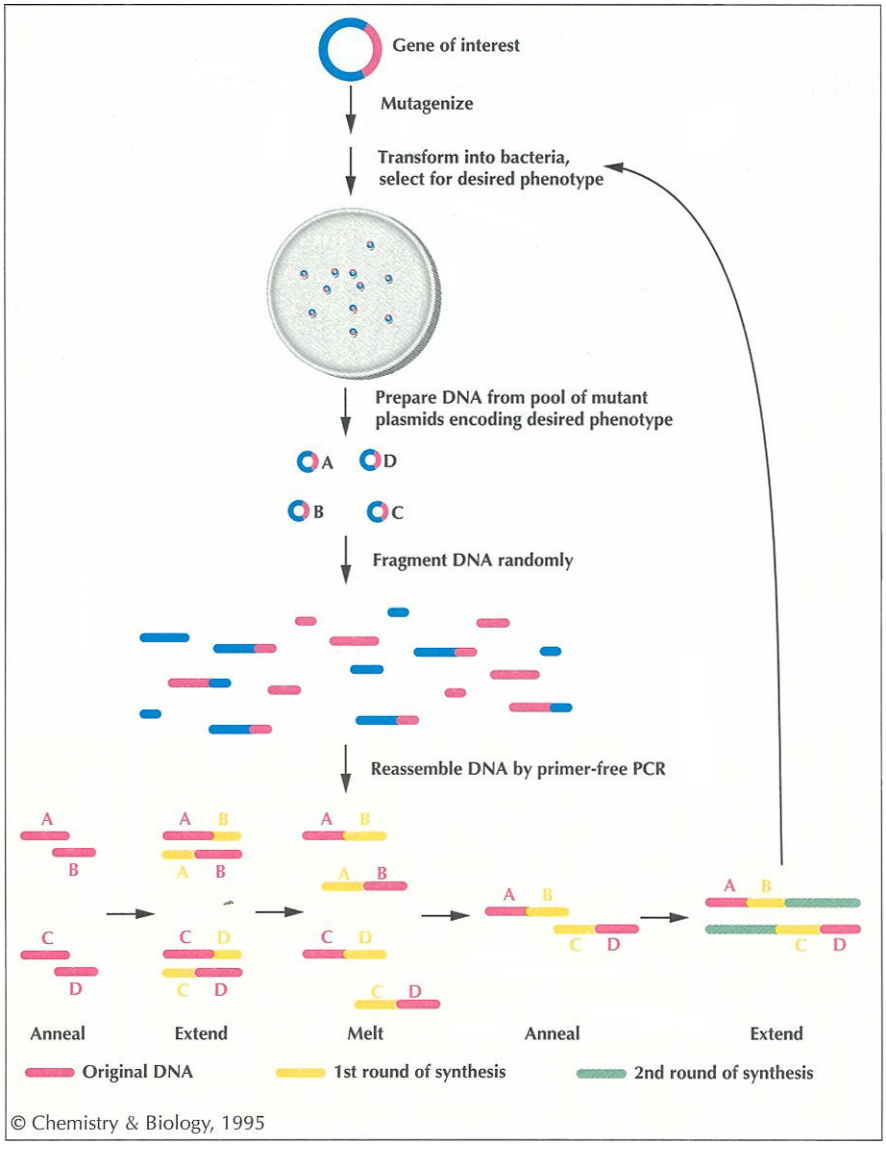

Fig. 3. *In vitro* evolution by artificial DNA recombination — the basic steps of DNA shuffling. The figure shows how independent mutations A, B, C and D can be combined by this technique. In the primer-free PCR reaction the original DNA is shown in magenta, DNA from the first round of synthesis in yellow and DNA from the second round of synthesis in green. For details see text and [19].

reactions catalyzed by enzymes encoded on different catabolic plasmids, and between the enzymes themselves. As well as PCBs discussed above, toluene is metabolized by a pathway encoded on one such plasmid. As with the range of PCB congeners that will be oxidized by a bacterium, substrate specificity in the toluene pathway depends in part on the dioxygenase catalyzing the first oxidation [7].

Biphenyl and toluene dioxygenases are four-subunit proteins that are so well conserved that functional dioxygenases are formed in *E. coli* containing a combination of components from the toluene (*tod*) and biphenyl (*bph*) operons. Components and reactions of the dioxygenases are shown in Figure 4. TodC1 (the large subunit of the terminal dioxygenase) combined with BphA2, BphA3 and BphA4 can oxygenate toluene, whereas the enzyme containing the original Bph1 cannot. Furthermore, expression of the TodC1 subunit in pseudomonads containing the *bph* operon enables them to grow on toluene, showing that once toluene has been oxidized its mineralization can be catalyzed by Bph enzymes [7].

Toluene dioxygenase can also oxidize the non-aromatic substrate trichloroethylene [23], whereas the biphenyl enzyme cannot. It is both surprising and interesting that two of the Tod/Bph hybrid enzymes degrade trichloroethylene more rapidly than the original toluene dioxygenase (Fig. 4c) [24]. Thus the modular nature both of the pathway and of individual enzymes means that by simply recombining existing structural information, an enzyme with new catabolic properties can easily be generated.

There is no reason in principle why recombinogenic mutation strategies should be limited to individual enzymes. Since enzymes acting in a single pathway are often encoded on the same plasmid, the entire plasmid can be subjected to DNA shuffling-like procedures. This should allow the evolution of an entire pathway to accommodate a new substrate without having to worry about which step is rate-limiting, which might easily be different for different substrates. The only requirement is that, for every section of a pathway that is to be subjected to evolutionary modifications, there must be a selectable phenotype.

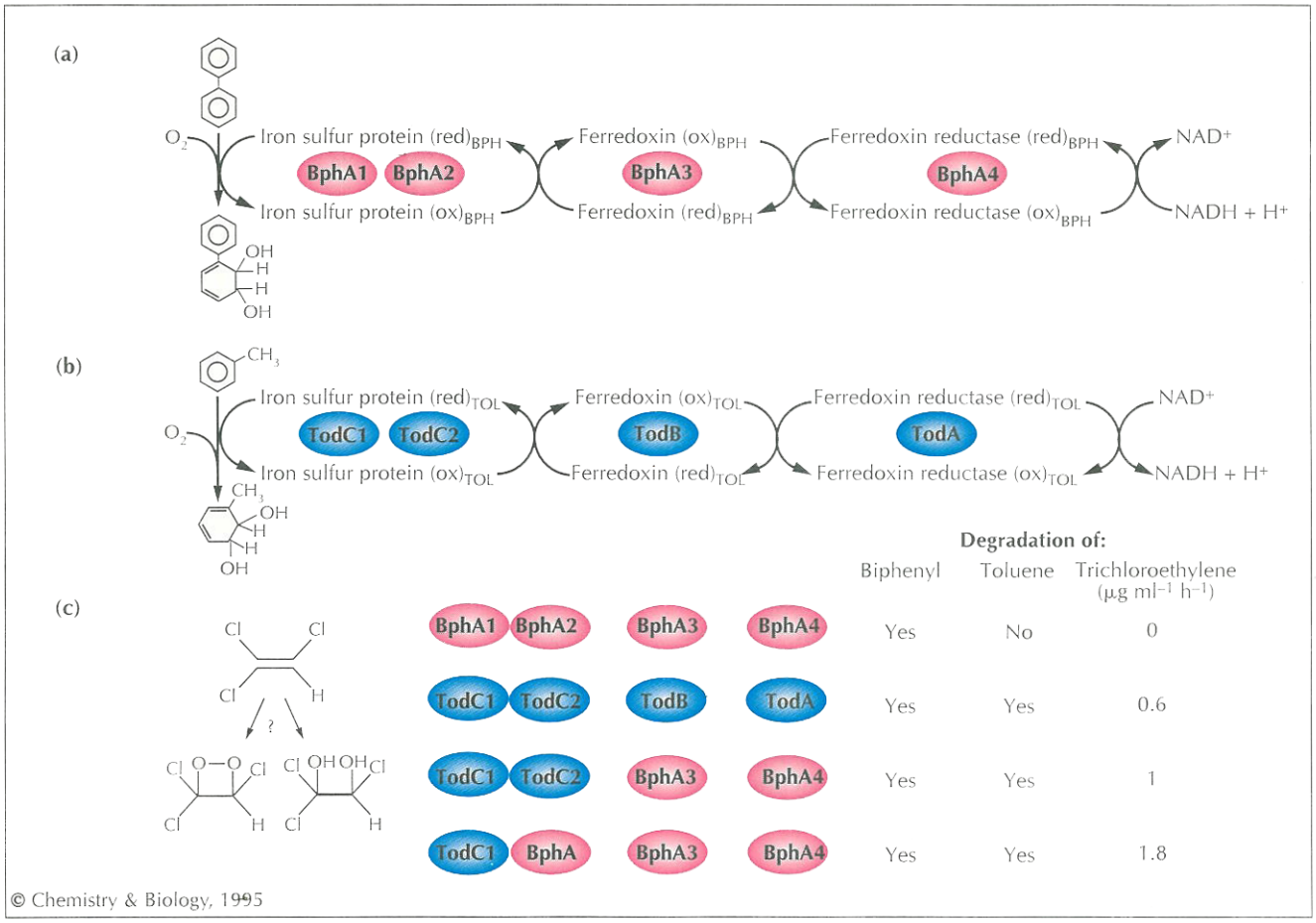

Fig. 4. Activities of hybrid dioxygenases against substituted aromatic hydrocarbons and trichloroethylene. **(a)** The degradation of biphenyl begins with a dioxygenation catalyzed by a four-subunit enzyme encoded by the *bph* operon. **(b)** The degradation of toluene begins with a similar dioxygenation catalyzed by a similar four-subunit enzyme encoded by the *tol* operon. **(c)** Hybrid *Pseudomonas* dioxygenases were made by expression of different combinations of genes from the *tol* and *bph* operons. The abilities of the hybrid enzymes to oxygenate biphenyl, toluene and trichloroethylene are indicated [23,24].

Combining pathways from different organisms

Genetic exchange has already been exploited to achieve biodegradation of the herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T). Based on the observation of cross-hybridization between plasmids carrying genes for catabolism of toluene, salicylate and chlorobenzoate, bacteria harboring a variety of catabolic plasmids were incubated in a chemostat with increasing concentrations of 2,4,5-T. After 8–10 months a culture developed that was able to use the herbicide as a sole carbon source [25].

In this case the xenobiotic was not degraded by a single bacterial species but by a consortium of microorganisms. This highlights an important consideration in designing biodegradative pathways: the enzymes required to metabolize a particular compound may not exist in a single microorganism. Natural bacterial populations exchange not only genetic information but also nutrients [12]. Some xenobiotics resembling a natural substrate are initially attacked by enzymes in one organism but are converted to 'dead-end' compounds that the microorganism cannot use because it lacks enzymes that recognize the product of the initial reaction. These compounds may diffuse into a second microorganism that is able to metabolize them further, obtaining energy or a required element from them. Clearly in the case of such co-metabolism there is no selection for improvement in the rate of the first enzyme, since the xenobiotic is recognized by the first microorganism only serendipitously, and only the second microorganism derives any benefit from its degradation.

Selection for improved enzymes in these cases is still possible if metabolic pathways can be constructed in a single host organism. It is even possible to combine parts of a pathway that normally occur in aerobic organisms with those that take place in anaerobes, as has been done for the degradation of pentachloroethane [26]. Pathways have also been combined by forced matings between different genera; crossing a species able to metabolize biphenyl with one that could use 3-chlorobenzoate resulted in a bacterium able to degrade 3-chlorobiphenyl [27].

Design and chance

There are obviously limitations to the extent to which metabolic pathways can be combined. Simultaneous expression of *Pseudomonas* biphenyl- and chloroben-

zoate-metabolizing enzymes to produce PCB-degrading bacteria can be disastrous because the products of one pathway inhibit enzymes in the other. For example, oxygenation of 3-chlorobenzoate yields 3-chlorocatechol. This is normally cleaved by the *ortho*-fission pathway (Fig. 1). If the biphenyl-metabolizing *meta*-fission pathway is also present, however, 3-chlorocatechol can be cleaved by an extradiol dioxygenase yielding an extremely reactive acyl chloride. This covalently modifies amino acids in the active site of the enzyme that formed it, thereby inactivating the enzyme [28].

Developing new xenobiotic catabolic activities will therefore depend equally on understanding the metabolic pathways involved and on being able to redirect the activities of individual enzymes. Isolation of different microorganisms from heavily polluted areas often reveals alternative strategies for metabolizing the same compounds. Information on the details of known metabolic pathways can be found on the World Wide Web Biodegradation site recently set up by Lynda Ellis and Lawrence Wackett (http://dragon.labmed.umn.edu/~lynda/), which will be an important new tool in metabolic pathway design. This facility should enable researchers not only to engineer enyzmes involved in xenobiotic degradation, but perhaps also to divert catabolic pathways for the synthesis of industrially desirable compounds.

The politics of pollution

Naturally the ability of genetically engineered microorganisms to transform man-made pollutants into harmless or useful products is only a part of the solution. There are additional questions, both microbiological and political, whose answers will determine the success of bioremediation as an industrial cleaner. Do we really want to release genetically engineered microorganisms into the environment, and if so will it be possible for these modified bacteria to compete in the wild? Bioreactor treatment of industrial effluents could avoid the problem of modified microorganism release, and would have the advantage that one would be working with a relatively defined mixture of compounds that have not had the opportunity to react with components of the soil. Pollutants that have already been released present more of a problem as they move up the food chain. Will the free market provide support for research in bioremedial technologies, or will it be necessary to pass legislation like that recently enacted by the Dutch government, providing strict penalties for polluters, to stimulate sufficient economic interest?

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