# Bacteria designed for bioremediation

Kenneth N. Timmis and Diemar H. Pieper

Although many environmental pollutants are efficiently degraded by microorganisms, others persist and constitute a severe health hazard. In some instances, persistence is a consequence of the inadequate catabolic potential of the available microorganisms. Gene technology, combined with a solid knowledge of catabolic pathways and microbial physiology, enables the experimental evolution of new or improved catabolic activities for such pollutants.

Environmental pollution can harm the flora and fauna of affected habitats, result in the uptake and accumulation of toxic chemicals in food chains, and, in some instances, cause serious health problems and/or genetic defects in humans. Although substantial progress has been made in reducing chronic industrial pollution over recent years, major accidents still occur, a considerable number of known polluted sites exist and new ones are continually being discovered. Some of these sites are or threaten to become sources of contamination of drinking-water supplies, and thereby constitute a substantial health hazard for current and future generations.

The public has a right to be protected from such hazards and it is imperative that critical cases of pollution be remedied as quickly as possible. Bioremediation constitutes an attractive alternative to physicochemical methods of remediation, or removal to 'safer' sites. In particular, it can be less expensive, can selectively achieve complete destruction of organic pollutants without collateral destruction of either the site material or its flora and fauna, and can be used *in situ* for pollutants that are present at low but environmentally relevant concentrations. Although some instances of pollution can be readily bioremediated using existing technology, those involving toxic, chemically stable compounds such as polychlorinated biphenyls (PCBs) or chlorinated dibenzo-*p*-dioxins require the development of new, innovative technologies.

## The need for designed organisms and consortia

A large number of microorganisms have been isolated in recent years that are able to degrade compounds previously considered to be non-degradable. This suggests that, under the selective pressure of environmental pollution, a microbial capacity for the degradation of recalcitrant xenobiotics is developing that might be harnessed for pollutant removal by biotechnological processes. Nevertheless, the fact that many pollutants persist in the environment emphasizes the current inadequacy of this catabolic capacity to deal with such pollutants.

There are a number of factors, some non-biological, that contribute to the persistence of pollutants in the environment, one of which is the fact that current pathways for the metabolism of xenobiotics are not optimal. This is particularly true for highly toxic pollutants such as dioxins, dibenzofurans and PCBs, for which effective pathways have not yet been described. There is thus a need to accelerate evolutionary developments by experimentally designing new pathways. Moreover, most microbial activities that can serve as the basis of biotechnological applications do not function optimally under process conditions and can almost always be improved.

The design of improved biocatalysts involves different aspects of optimization, including: creating new (so far undescribed) metabolic routes; expanding the substrate ranges of existing pathways; avoiding substrate misrouting into unproductive routes or to toxic or highly reactive intermediates; improving the substrate flux through pathways to avoid the accumulation of inhibitory intermediates (e.g. catechols); increasing the genetic stability of catabolic activities; increasing the bioavailability of hydrophobic pollutants; and improving the process-relevant properties of microorganisms[1].

The rational optimization of known catabolic activities and the creation of new activities relies on the extensive and rapidly growing knowledge base of pathway genetics, biochemistry and structure–function relationships of the component enzymes that has been generated over many years by a large body of dedicated, devoted and enthusiastic scientists. Accessing and exploiting this knowledge has been considerably facilitated recently through establishment of the University of Minnesota Biocatalysis/Biodegradation Database (L. P. Wackett and L. B. M. Ellis; http://www.labmed.umn.edu/umbbd/).

## *In vivo* and *in vitro* design strategies

A variety of strategies for designing new or improved catalysts for bioremediation have been developed over recent years. The simplest strategy is improving the biodegradative performance of a consortium (a mixed bacterial culture) through the addition of a 'specialist' organism; in this case, a consortium is designed[2–5]. Consortia that exhibit novel catabolic activities can also be obtained by sustained selective pressure in, for example, a chemostat. Here, although it is not possible to talk about design in the sense of the deliberate combination of known genetic elements to achieve a desired phenotype, the design of the selection system results in the desired phenotype.

In some cases, a simple two- or three-member consortium is obtained, one member of which may carry

*K. N. Timmis (kti@gbf.de) and D. H. Pieper (dpi@gbf.de) are at the Division of Microbiology, GBF – National Research Centre for Biotechnology, Braunschweig, Germany.*


0167-7799/99/$ – see front matter  PII: S0167-7799(98)01295-5

out the initial catabolic reactions and another of which may complete the sequence. Such consortia have been developed for the mineralization of bicyclic aromatics such as chlorinated biphenyls[6], chlorinated dibenzofurans[3] and aminonaphthalenesulfonates[5]. In these examples, one member of the consortium transforms the subtrate into the corresponding chlorinated benzoate or salicylate and grows at the expense of the initially attacked aromatic ring; a second member then mineralizes the formed benzoate or salicylate. These consortia are restricted to congeners that can be initially transformed by a biphenyl or dibenzofuran dioxygenase.

An alternative approach for the mineralization of highly chlorinated congeners is the development of anaerobic–aerobic processes. The discovery that microbial degradation of PCBs occurs in sediments and that anaerobic dehalogenation is enhanced by an increase in halogen substitution (in contrast to aerobic degradation, for which the persistence generally increases with increasing halogen substitution) brought the hope that this process could be used to transform highly chlorinated biphenyls into less-chlorinated congeners, which are more amenable to aerobic degradation.

However, only a few cultures able to dechlorinate PCBs reductively have been obtained to date[7,8]. By contrast, microbial isolates such as *Desulfumonile tiedjei*[9], which are able to dehalogenate chlorobenzoates or chlorophenols reductively[10] ('halo-respiration'), are available. The complete mineralization of the persistent herbicide 2,3,6-trichlorobenzoate by the combined activity of anaerobic and aerobic bacteria in an appropriately designed bioreactor has also been reported[11]. This suggests that combined anaerobic–aerobic processes may be promising for the complete mineralization of PCBs and even chlorinated dioxins.

The metabolic 'division of labour' in co-cultures of aerobic microorganisms may not constitute the most effective situation and prolonged selection may lead to the transfer of genetic determinants of catabolic functions between members of the consortium and the emergence of a single organism with the complete catabolic sequence. Such natural gene-transfer events are the basis of numerous *in vivo* design experiments[12–18] and are facilitated by the fact that naturally occurring pathways for the metabolism of organic compounds are often encoded by broad-host-range plasmids[19–21].

In most cases, the conjugal transfer of a catabolic plasmid from its original host to an appropriate recipient results in the combination of a central pathway (e.g. the chlorocatechol *ortho*-cleavage pathway[18] for the ring cleavage of aromatic compounds and channelling the products to Krebs-cycle intermediates) with a pathway sequence that enables a new substrate to be channelled into the central pathway. This approach does not always succeed, in some instances because the relevant genes are not on a transmissible plasmid or are located on a narrow-host-range plasmid. Even if it does succeed, the new phenotypes may not be stable, because the plasmids on which the catabolic genes reside often segregate in the absence of selective pressure.

Gene cloning generally circumvents barriers to natural gene transfer[22,23] and, of course, involves precisely predetermined genes and expression signals. Plasmid cloning vectors may, however, suffer from the same instability as natural plasmids and, moreover, have antibiotic-resistance selection markers, which are undesirable for environmental applications. For these reasons, minitransposon cloning vectors have been developed to insert heterologous genes stably into the chromosomes of host bacteria without the use of antibiotic-resistance markers[24,25] or, more recently, with markers that can be selectively eliminated after gene transfer[26]. As the transposase gene is not cotransferred, the transposon vectors do not cause sequence instability or rearrangements at the site of transposition, nor do they mediate immunity to transposition. They can therefore be used for multiple, sequential cloning events in the same host organism.

**Improving catalyst performance**

Some bioremediation processes require an increase in the rate of pollutant removal. Achieving this goal involves identifying the enzymatic or regulatory step of the pathway that is rate limiting, followed by experimental elevation of the activity of the rate-limiting protein through an increase in the transcription or translation of its gene, or in its stability or kinetic properties. Transcription of the genetic determinants of metabolic pathways, which are usually organized in operons, is generally controlled by positively-acting regulatory proteins that are activated by pathway substrates or metabolites[27–29].

Mutants of regulatory proteins have been produced that either mediate higher levels of transcription than the wild-type regulator or respond to new effectors[30–32]. The use of artificial regulatory systems allows the expression of catabolic genes to be uncoupled from the signals that ordinarily control their expression and offers considerable flexibility for process control. Where metabolism of the pollutant is not energy yielding (co-metabolism) and/or where induction requires the addition of exogenous inducers that may themselves be toxic, as in removal of trichloroethylene (TCE) in contaminated ground-water samples[33–35], the use of artificial regulatory systems or constitutive expression signals may be helpful. The use of starvation-induced promoters can uncouple gene expression from growth and augment catabolic activity in nutrient-limited environments or when target pollutants fall below certain thresholds[36].

Protein engineering can be exploited to improve an enzyme's stability, substrate specificity and kinetic properties. Rational design of proteins performed by site-directed mutagenesis requires an understanding of structure–function relationships in the molecule and therefore a detailed knowledge of the three-dimensional structure of the enzyme itself[37], or of at least one member of the protein family, to allow the structure of the protein under study to be modelled. However, the number of degradative enzymes whose structure has been elucidated is still small and this constitutes a major limitation for rational protein design (but see Ref. 38 for elegant examples of the power of this approach if structural information is available). Where phenotypic selection of desired variants is possible, rare spontaneous or induced mutants may be readily obtained; where phenotypic selection of variants is not possible, other, more-efficient approaches are needed.

One approach to combining the best attributes of related enzymes is to exchange subunits or subunit

sequences. For example, enzyme variants with superior TCE-transformation kinetics were obtained by exchanging subunits between the multicomponent toluene and biphenyl dioxygenases[39]. Further experiments to exchange domains of the α subunits of biphenyl or (chloro)benzene dioxygenases exhibiting different substrate specificities resulted in chimeric enzymes with broader substrate specificities than the parental enzymes[40–43]. A more recently developed and powerful alternative method for obtaining proteins with new activities involves shuffling their gene sequences[44,45].

**The need for complete pathways**

In some cases, although a complete pathway for a particular substrate may not exist in a single organism, partial and complementary pathway segments may exist in different organisms. The development of an organism exhibiting a desired catabolic phenotype may therefore require the combination of determinants for complementary pathway segments in order to form a complete pathway sequence for a target substrate.

Complete metabolic pathways may be needed for two reasons. First, co-metabolic processes need an input of energy and therefore represent a metabolic burden for the microorganism. Second, the end metabolites produced by incomplete pathways may be toxic or subject to further transformations by other microorganisms, forming reactive or toxic molecules.

One example of this is found in PCB metabolism, in which microorganisms usually metabolize only one aromatic ring and accumulate the others as the corresponding chlorobenzoates, which have been shown to be inhibitory[46,47]. The metabolites formed from the further metabolism of chlorobenzoates by benzoate pathways of indigenous microorganisms are even more problematic. 3-Chlorocatechol is a known suicide substrate of *meta*-cleavage enzymes such as 2,3-dihydroxybiphenyl dioxygenase[48], which is involved in the transformation of chlorobiphenyls to chlorobenzoates and can thereby directly inhibit further PCB metabolism. Further metabolism of chlorocatechols via the 3-oxoadipate pathway can result in the formation of the antibiotic protoanemonin[49], which may cause a rapid decline in viable PCB-transforming microoganisms[6].

**'New' metabolic routes**

If a productive metabolic route within an organism or community for a pollutant is not known, or known routes are ineffective, the challenge is to design and create a 'new' effective pathway ('new' meaning that we have not yet found such a pathway in nature, not that it does not exist). In the past, the strategy for designing new pathways was based on the judicious combination of partial metabolic sequences of known routes, coupled with a search for enzymes to complete the channelling of intermediates into central pathways. One example of this is the patchwork assembly of a new pathway for the mineralization of alkylaromatics[23].

The reason for creating this route was the problematic degradation of mixtures of chloro- and alkylaromatic pollutants, which ordinarily induce expression of both *ortho*- and *meta*-ring-cleavage pathways and thereby cause the misrouting of central catechol intermediates into unproductive routes. This results in the death of the microbial community. The designed strain contains two parallel *ortho*-cleavage pathways but no *meta*-cleavage activity, and can simultaneously catabolize chloro- and alkylaromatics. This designed strain not only grows well on ordinarily toxic substrate mixtures but, in so doing, also protects other members of the microbial community[50,51].

Although there are few examples in the literature of the metabolic design of 'new' pathways, this approach has great scientific interest and the potential for novel applications. The University of Minnesota Biocatalysis/Biodegradation Database should soon offer a systematic display of theoretical routes from one substrate to a specific intermediate or central metabolite. This will, in principle, not only permit multiple options to be considered and tested but also expose truly 'novel' possibilities.

**Improving pollutant bioavailability**

Bioremediation is limited not only by the recalcitrance of the target pollutants as a result of their chemical stability but also by the toxicity of such compounds and, in particular, the limited bioavailability of hydrophobic, poorly water soluble pollutants such as PCBs. Biological reactions occur in or at the interface of the aqueous phase and surfactants have the ability to desorb and disperse poorly soluble compounds in small, high-surface-area micelles within the water phase. Surfactants can thus improve the accessibility of these substrates to microbial attack[52]. The high surface activity, heat and pH stability, low toxicity and biodegradability of biosurfactants constitute important advantages over synthetic surfactants, particularly for environmental applications. Despite the fact that application of biosurfactants has been shown potentially to increase the degradation rate of hydrophobic pollutants[53], the high cost of biosurfactant production restricts their application. Current efforts are therefore directed towards the design of recombinant biocatalysts that exhibit a desired catabolic trait and produce a suitable biosurfactant[54].

**Improving catalyst survival in the environment**

It is sometimes assumed that a major problem in the use of designed inoculants is their poor competitiveness in natural environments. In fact, a number have been shown to survive well in a variety of natural settings[51,55]. Nevertheless, improving inoculant survival is an important goal in the further development of bacterial inocula for biotechnological applications in the environment[56], where the microorganisms are exposed to a variety of stresses such as toxic metals, solvents and extremes of temperature and pH.

The combination of resistance to environmental stresses and catabolic phenotypes in appropriate bacterial strains is expected to yield microbial catalysts with significantly improved survival characteristics in hostile habitats. For example, solvent-resistant bacteria able to mineralize hydrophobic pollutants have recently been engineered[57,58]. Furthermore, there are numerous waste sites that are contaminated with mixtures of toxic chlorinated solvents and radionuclides, and only radiation-resistant microorganisms can survive in such environments. Strains of *Deinococcus radiodurans* expressing toluene dioxygenase have now been constructed that are able to transform the target compounds in highly radioactive environments, and these are promising biocatalysts for lowering the toxicity of such sites[59].

The rhizosphere is a potential habitat for the degradation of pollutants because it provides a selective advantage to those microorganisms adapted to its unique environment, and substantial carbon and energy to support pollutant-degrading activity. Rhizosphere pseudomonads have been engineered to transform PCBs, TCE or dichlorobenzoate[60–62]. A logical extension of the philosophy of providing a selective advantage for the catalyst is the development of 'field application vectors': bacteria able to degrade the chemical detergents that are applied with the bacteria. In this case, the detergents not only provide a selective advantage but may also increase the bioavailability of hydrophobic target pollutants[63].

## Conclusions

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

## Acknowledgments

Work by the authors has been supported by grants from the German Ministry of Education and Research, and the European Union. K. N. Timmis expresses gratitude to the Fonds der Chemischen Industrie for their generous support.

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