Engineering of improved microbes and enzymes for bioremediation

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Bioremediation with microorganisms is an attractive alternative to conventional techniques, such as incineration and chemical treatment, for disposing of pollutants. Recent progress in molecular biology, microbiology, and genetics is providing the driving force towards engineering improved microbes and enzymes for bioremediation. A number of genetic engineering approaches have been developed in the past several years that have proven useful in introducing/evolving the desired properties for different biodegradative pathways or enzymes. The initial excitement generated in this area should continue to pave the way for rational or irrational design of microbes or enzymes with novel remedial properties.

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Abbreviations

3-MP	3-methylpentane
MT	metallothionein
OPH	organophosphorus hydrolase
PCB	polychlorinated biphenyl
TCE	trichloroethylene

Introduction

Over the past few decades enormous quantities of industrial pollutants have been released into the environment. A large number of them, particularly those structurally related to natural compounds, are readily degraded or removed by microorganisms found in soil and water. Superimposed on the rich variety of pollutants present in the environment, however, is an increasing number of novel industrial compounds not found extensively in nature. These xenobiotic compounds are usually removed slowly and tend to accumulate in the environment. Due to the high degree of toxicity, their accumulation can cause severe environmental problems.

Because of the problems associated with pollutant treatment by conventional methods, such as incineration or landfill, increasing consideration has been placed on the development of alternative, economical and reliable biological treatments. Although natural microorganisms collectively exhibit remarkable evolutionary capabilities to adapt to a wide range of chemicals, natural evolution occurs at a relatively slow rate, particularly when the acquisition of multiple catalytic activities is necessary. In such cases, the acceleration of these events via genetic/metabolic engineering [1] may be helpful as specific genetic alterations can be carefully designed and controlled.

Recent advances in molecular biology have opened up new avenues to move toward the goal of engineering microbes or enzymes to function as 'designer biocatalysts' [2–4], in which certain desirable traits from different organisms are brought together with the aim of performing specific bioremediation. In this review, we will focus on advances of the past two years with emphasis on new approaches to engineering improved microbes or enzymes for bioremediation.

Engineering microbes for improved bioremediation

Bioadsorbents for heavy metal removal

The discharge of heavy metals from agricultural, industrial, and military operations has serious adverse effects on the environment. Conventional technologies, such as precipitation-filtration, ion exchange, reverse osmosis, oxidation-reduction, and membrane separation, are often inadequate to reduce heavy metal concentrations in wastewater to acceptable regulatory standards. Recent research has focused on the development of novel bioadsorbents with increased affinity, capacity, and selectivity for target metals.

Higher organisms respond to the presence of heavy metals with the production of metallothioneins (MTs). MTs are cysteine-rich proteins that bind metal ions (cadmium, lead, mercury and copper) and sequester them in biologically inactive forms [5]. Expression of MTs in *Escherichia coli* to improve the bioadsorption of heavy metals is a promising technology for the development of microbial-based biosorbents. Metal removal by intracellular MTs has been problematic, however, because of limited metal uptake. One promising approach to circumvent this problem is the expression of MTs on the cell surface [6•]. Sousa and co-workers demonstrated this possibility by inserting MTs in the permissive site 153 of the LamB sequence. Expression of the hybrid proteins increased Cd^{2+} binding by 15–20 fold. As the MTs are displayed on the cell surface, it is conceivable that even non-viable cells could be used for metal accumulation and desorption. In the future, expression of MTs on the surface of other environmentally robust organisms, such as *Pseudomonas*, could prove to be a very promising strategy.

An alternative solution to overcome the deficiencies of metal uptake is to simultaneously express the corresponding metal transport system with MTs. Chen and Wilson [7•,8] demonstrated this feasibility by coexpressing MTs together with the Hg^{2+} transport proteins MerT and MerP in *E. coli*. The subsequent bioaccumulation of Hg^{2+} was increased significantly. Similar enhancements in metal

bioaccumulation may be possible by coexpressing other metal transport systems with MTs.

The *de novo* design of metal-binding peptides is an attractive alternative to MTs as they offer the potential of better affinity and selectivity for heavy metals [9,10]. Peptides with an abundance of cysteine residues, for example, are known to bind Cd^{2+} and Hg^{2+} with a very high affinity. Expression of a repetitive metal-binding motif, $(Cys–Gly–Cys–Cys–Gly)_3$, as a fusion with the maltose-binding protein in *E. coli* enhanced Cd^{2+} and Hg^{2+} binding by 10-fold. Cells expressing the peptide fusion effectively and selectively removed Cd^{2+} and Hg^{2+} at parts per billion concentrations [11]. The ability to incorporate multiple binding sites within a single peptide for several different metals may prove to be a versatile strategy for the removal of mixed metal wastes.

Novel metal-binding peptides with high affinities for cadmium have been selected from a phage–display library [12]. The peptide His–Ser–Gln–Lys–Val–Phe, which exhibits the strongest relative affinity for Cd^{2+}, was cloned into *E. coli* as a fusion to the cell-surface-exposed area of the outer membrane protein OmpA. Cells expressing this peptide showed increased survival in growth medium containing toxic levels of $CdCl_2$, demonstrating the binding of Cd^{2+} by the surface-exposed peptide. Similarly, cells expressing hexaHis peptides as a fusion to the LamB protein showed a fivefold increase in cadmium accumulation for one hexaHis peptide, and an 11-fold increase when two hexaHis peptides were expressed in tandem [13]. It is clear that the expression of multiple tandem repeats of the metal-binding motif may further increase the overall accumulation of heavy metals.

Designing strains for enhanced biodegradation

Many environmental pollutants are readily degraded by naturally-occurring microbes. Very often, however, the rate of degradation may not be optimal for practical large-scale bioremediaton. Genetic engineering of biodegradative pathways offers the potential of expanding the existing capabilities found in nature. Several notable, recently-published examples will be discussed.

Organophospates are used extensively as agricultural and domestic pesticides and are one of the most toxic compounds known. Organophosphorus hydrolase (OPH) isolated from soil microorganisms has been shown to degrade these pesticides effectively. The use of OPH for detoxification, however, has always been limited by the high cost associated with purification. Whole-cell detoxification is more cost effective; however, it is also limited by the transport barrier of organophosphates across the cell membrane [14]. Surface expression of OPH can circumvent transport limitations imposed by cell membranes in much the same way that surface expression of MTs enhanced the metal-binding capability of cells [15••]. Whole cells expressing OPH on the cell surface degraded parathion and paraoxon sevenfold faster compared to whole cells expressing OPH intracellularly. The resulting live biocatalysts were also considerably more stable and robust than purified OPHs, retaining 100% activity over the 30 day period studied when maintained at 37°C [14]. Immobilization of these novel biocatalysts onto solid supports provides an attractive means for pesticide detoxification in place of immobilized OPH [16].

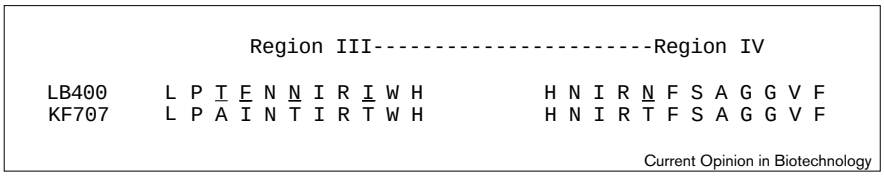
Expression of biocatalytic pathways in foreign microorganisms can significantly enhance the efficiency of the biodegradation process. One such example was recently reported for the desulfurization of fossil fuels. A number of organisms, such as *Rhodococcus erythropolis*, have been found to remove sulfur from fossil fuel via a sulfur-specific pathway, selectively cleaving the sulfur without ring destruction and therefore maintaining the fuel content [17]. The catabolic genes *dszA*, *B*, and *C*, responsible for desulfurization have been cloned and characterized [18]. *Pseudomonas* strains have been engineered by inserting the *dsz* cluster into their chromosomes [19•]. These recombinants desulfurized dibenzothiophene more efficiently than the native *R. erythropolis* IGTS8. Furthermore, by expressing the desulfurization pathway in *Pseudomonas aeruginosa* EGSOX, the ability to carry desulfurization and to produce rhamnolipid, a biosurfactant that increases the aqueous dibenzothiophene concentration, was effectively combined into a single strain. The resulting recombinant degraded dibenzothiophene four times faster than the wild-type *R. erythropolis*. The design of novel biocatalysts endowed with a desulfurization phenotype offers new insights for the development of commercially viable desulfurization processes.

The expression of detoxifying enzymes in foreign organisms may also confer specific selective advantages for *in situ* applications. This strategy was recently reported using a wheat rhizosphere system for the detoxification of soil-borne trichloroethylene (TCE) [20]. The toluene *o*-monooxygenase genes of *Burkholderia cepacia* G4 were engineered into *Pseudomonas fluorescens* 2-79. *P. fluorescens* colonizes wheat roots better than other known colonizers, enabling the establishment of a bacterium–plant–soil microcosm. Treatment of TCE-contaminated surface and near-surface soil was demonstrated, with more than 63% of the initial TCE removed within four days. The most attractive aspect of this technology is its low cost, as only expenses required for planting are necessary.

A recent survey of Department of Energy waste sites indicates that about 32% of soils and 45% of groundwaters are co-contaminated with organopollutants and radionuclides. As most microorganisms are sensitive to the damaging effects of radiation found in mixtures containing radionuclides, a different bioremediation strategy is necessary. *Deinococcus radiodurans* is a soil bacterium that can survive acute exposures to ionizing radiation of 15,000 Gy without lethality [21]. A recombinant *D. radiodurans* strain expressing the toluene dioxygenase from *Pseudomonas putida* F1 was shown to effectively oxidize toluene, chlorobenzene, and TCE in a highly irradiating environment [22•]. The recombinant strains were

Figure 1

Comparison of region III and IV of the large subunit of biphenyl dioxygenases from *B. cepacia* LB400 and *P. pseudoalcaligens* KF707. Sequences underlined in LB400 are reported to be critical for substrate specificity.



also tolerant to the solvent effects of toluene and TCE at levels exceeding those of many radioactive waste sites. The prospect of using this strategy to treat a variety of organic wastes in the presence of radionuclides is very promising.

Protein engineering for improved biodegradation

Many wild-type microorganisms are capable of transforming, or in some cases completely mineralizing, man-made compounds through biodegradative pathways that were evolved for naturally occurring compounds of similar structure. The ever-increasing information regarding the structure and function of enzymes and pathways involved in biodegradation of recalcitrant pollutants offers opportunities for improving enzymes or entire pathways by genetic engineering. Control mechanism and enzyme properties can be tailored by site-directed mutagenesis which is often guided by computer-assisted modeling of the three-dimensional protein structures. For example, site-directed approaches have been recently applied to engineer the active-site topology of cytochrome P450 [23], to enlarge the binding pocket of haloalkane dehalogenase [24], and to influence the substrate range of aromatic dioxygenases.

Although camphor is the natural substrate for cytochrome $P450_{cam}$, this enzyme has a low level of activity towards other small hydrophobic molecules such as styrene, ethylbenzene and some small alkanes. By engineering its active site volume and topology, Stevenson *et al.* [23] enhanced the activity, substrate specificity and regioselectivity of $P450_{cam}$ for hexane and 3-methylpentane (3-MP). Reducing the active-site volume by the Tyr96→Ala–Val247→Leu double mutation resulted in fourfold higher activity for the oxidation of hexane over 3-MP. Whereas increasing the active-site volume by the Tyr96→Ala and Tyr247→Ala double mutation generated an enzyme with a twofold preference for 3-MP over hexane. Thus engineering of the active site volume can be used to tune enzymes for desired substrate specificities and regioselectivities.

Xanthobacter autotrophicus can utilize 1,2-dichloroethane as its sole carbon source. Haloalkane dehalogenase is the initial enzyme responsible for the substitution of a terminal chlorine atom by a hydoxyl group. The catalytic mechanism of this reaction is well understood and has been shown to dechlorinate a wide range of substrates. Using structural information available for the enzyme–substrate, enzyme–intermediate, and enzyme–product complexes [25,26] as a guide, the bulky amino acids lining the catalytic cavity were replaced with alanine (increasing active-site volume). The resulting variants were severalfold more active in dechlorinating dichlorohexane [24]; however, no mutant tested could utilize more bulky substrates such as TCE.

The biphenyl dioxygenases of strains *Burkholderia cepacia* LB400 (LB400) and *Pseudomonas pseudoalcaligenes* KF707 (KF707) are structurally very similar but exhibit different specificities for polychlorinated biphenyls (PCBs). Guided by sequence comparison, Mondello *et al.* [27] altered several amino acid residues within regions III and IV (Figure 1) of the large subunit of LB400 biphenyl dioxygenase to mimic those in KF707. Similarly, Kimura *et al.* [28] produced chimeric enzymes from the same wild-type dioxygenases by exchanging restriction fragments. With both approaches, variant enzymes were obtained with the capability to hydroxylate double *ortho*- and double *para*-substituted PCBs, thus combining the substrate range of both parental enzymes. The construction of hybrid nitrotoluene dioxygenases was also helpful in identifying the carboxy-terminal region of the large subunit of this enzyme as being critical for substrate specificity [29]. The recent resolution of the crystal structure of naphthalene dioxygenase [30] will undoubtedly be very helpful for tailoring aromatic dioxygenases by rational design.

Even with increasing information on structural and functional aspects of biodegradative enzymes, rational design approaches can fail due to unexpected influences exerted by the substitution of one or more amino acid residues. It is known that mutations far from the active site can modulate catalytic activity or substrate recognition but are difficult to predict *a priori*. Rational (site-directed) approaches are also restrictive because they allow the exploration of only a very limited sequence space at a time. Irrational approaches [31,32] such as DNA shuffling [33], random priming [34•] or staggered extension process [35•] can, therefore, be preferable alternatives to direct the evolution of enzymes or pathways with highly specialized traits. Similar to natural selection, in which multiple environmental forces select enzymes to meet a variety of challenges, irrational approaches do not require prior extensive structural or biochemical data. When combined with focused selection or screening, irrational approaches offer useful alternatives for generating both the desired improvements and a database for future rational approaches to protein design.

Perhaps the most powerful and promising utility of DNA shuffling is in the cross-breeding of genes between diverse classes of species because of the extended sequence space that can be explored [36••]. In two independent studies, the substrate range of biphenyl dioxygenases toward PCBs have been successfully extended using directed evolution [37•,38•]. Variants were obtained by random shuffling of DNA segments between the large subunit of two wild-type biphenyl dixoxygenases. Several variants had extended substrate ranges for PCBs exceeding those of the two parental enzymes. Molecular evolution is probably the most useful way for evolving enzymes with extended substrate specificities for PCBs and other recalcitrant compounds, because microbial degradation of xenobiotics is usually by incomplete co-metabolism and so does not exert a selective pressure on bacteria.

The optimization of an entire biodegradative pathway is more likely to be achieved by a directed evolution process than by rational design, which would depend on a vast set of often incomplete strucural and biochemical information on all the enzymes involved. This was recently demonstrated by the modification of an arsenic resistance operon using DNA shuffling [39•]. Cells expressing the optimized operon grew in up to 0.5 M arsenate, a 40-fold increase in resistance. Furthermore, a 12-fold increase in the activity of one of the gene products (ArsC) was observed in the absence of any physical modification to the gene itself. The authors speculate that modifications to other genes in the operon effect the function of the *arsC* gene product. Such unexpected but exciting results are more likely to be realized using irrational approaches.

Irrational strategies have also been employed to amplify homologous biodegradative enzymes and incorporate them into recombinant enzymes without isolation (or, in theory, knowledge of) the host microorganisms. This approach was recently demonstrated by the modification of catechol 2,3-dioxygenase (C23O) [40••]. Degenerate primers were used to amplify the central segment of C23O present in a consortia of microorganisms derived from soil and sea water samples. A second round of PCR incorporated the amplified central domains into 5′ and 3′ 'arms' of the *nahH* gene. Such an approach allows rapid exploitation of the natural sequence diversity already present in the environment for creation of novel hybrid enzymes or pathways with desired features.

Conclusions

Engineering microbes or enzymes for better bioremediation, until recently, has been successful mostly for a defined group of related problems. This has gradually been replaced, however, by either rational or irrational approaches that can now be applied to a wider range of pollutants. Although the ability to predictively design microbes or enzymes for any given remediation remains an overwhelming task, the increasing understanding of fundamental mechanistic principles generated from both basic research or directed evolution will probably lead to the emergence of novel solutions for improved bioremediation.

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