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Current Opinion in Biotechnology

Bioremediation: environmental clean-up through pathway engineering

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Given the immense risk posed by widespread environmental pollution by inorganic and organic chemicals, novel methods of decontamination and clean-up are required. Owing to the relatively high cost and the non-specificity of conventional techniques, bioremediation is a promising alternative technology for pollutant clean-up. Advances in bioremediation harness molecular, genetic, microbiology, and protein engineering tools and rely on identification of novel metal-sequestering peptides, rational and irrational pathway engineering, and enzyme design. Recent advances have been made for enhanced inorganic chemical remediation and organic chemical degradation using various pathway-engineering approaches and these are discussed in this review.

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Current Opinion in Biotechnology 2008, **19**:437–444

This review comes from a themed issue on
Tissue, Cell and Pathyway Engineering
Edited by William Bentley and Michael Betenbaugh

Available online 13th September 2008

0958-1669/$ – see front matter
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DOI 10.1016/j.copbio.2008.07.012

Introduction

Toxic inorganic and organic chemicals are major contributors to environment contamination and pose a major health risk to the human population. Prevention of future contamination from these compounds presents an immense technical challenge [1]. While various physico-chemical processes have been developed for treating these pollutants; these approaches are often prohibitively expensive, non-specific, or have the potential for introducing secondary contamination. As a result, there has been an increased interest in eco-friendly bio-based treatments commonly known as bioremediation. Bioremediation is often considered a cost effective and environmental friendly method and is gradually making inroads for environmental clean-up applications. Bioremediation relies on improved detoxification and degradation of toxic pollutants either through intracellular accumulation or via enzymatic transformation to less or non-toxic compounds [2]. Many microorganisms naturally possess the ability to degrade, transform, or chelate various toxic chemicals. However, these natural transformations are limited by the relative slow rates. Development of new genetic tools and a better understanding of microorganism's natural transformation ability at the genetic level are essential to accelerate the progress of designer microbes for improved hazardous waste removal. Several attempts have been made recently to enhance biotransformation and bioaccumulation of toxic wastes by microorganisms. This review will shed light on recent developments in designer microbes through pathway engineering.

Pathway engineering for enhanced bioremediation

Inorganic pollutant removal

Extensive environmental pollution by heavy metals and radionuclides (Table 1) primarily by anthropogenic origin is adversely affecting human health and environment and has led to stricter regulatory limits [3]. Although traditional methods are adequate for treating high concentrations of contamination, they are not cost effective at reducing the levels to regulatory limits [4]. Recently pathway-engineering techniques have been explored for selective and high capacity bioremediation of heavy metals and radionuclides.

Most microorganisms, when encountered with heavy metals, respond by producing metal-binding peptides such as phytochelatins (PCs) and metallothionein (MTs). These thiol-rich peptides bind to a variety of heavy metals and reduce their toxicity by sequestration [5]. These peptides have been extensively used to enhance the metal accumulation ability of microorganism by producing them in different subcellular locations. In one such effort, the *Arabidopsis thaliana* phytochelatin synthase (AtPCS) responsible for enzymatic PC synthesis was overexpressed in *E. coli*; leading to 20-fold higher heavy metal accumulation [6•]. Similar efforts were reported by Sriprang *et al.* [7] in a symbiotic *Rhizobia*

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Table 1

Inorganic chemicals and radionuclides

Contaminant	MCLG (mg/L)	MCL (mg/L)	Potential health effects from ingestion of water	Sources of contaminant in drinking water
Arsenic	0	0.010 as of 01/23/06	Skin damage or problems with circulatory systems, and may have increased risk of getting cancer	Erosion of natural deposits; runoff from orchards, runoff from glass and electronic production wastes
Cadmium	0.005	0.005	Kidney damage	Corrosion of galvanized pipes; erosion of natural deposits; discharge from metal refineries; runoff from waste batteries and paints
Lead	0	0.015	Infants and children: delays in physical or mental development; children could show slight deficits in attention span and learning abilities. Adults: kidney problems; high blood pressure	Corrosion of household plumbing systems; erosion of natural deposits
Mercury (inorganic)	0.002	0.002	Kidney damage	Erosion of natural deposits; discharge from refineries and factories; runoff from landfills and croplands
Radium 226/228 (combined)	0	5 pCi/L	Increased risk of cancer	Erosion of natural deposits
Uranium	0	30 μg/L as of 12/08/03	Increased risk of cancer, kidney toxicity	Erosion of natural deposits

MCLG: maximum contaminant level goal; MCL: maximum contaminant limit; Source: EPA safe water.

bacterium. While these reports demonstrated the potential of using PCs for heavy metal accumulation, a limited supply of the precursor glutathione (GSH) was a bottleneck in PC production and the subsequent heavy metal accumulation. One promising approach to alleviate the PC synthesis bottleneck is to co-express the enzymes responsible for GSH production. Kang *et al.* [8] demonstrated this feasibility by co-expressing the feedback desensitized glutathione synthase with PC synthase, resulting in 10-fold increase in PC production. However, Cd accumulation was only increased by 2-fold, leading to the speculation that Cd uptake could be a problem. This limitation was resolved by expressing a Cd uptake transporter MntA, and the final Cd accumulation of 31.6 μmol/g dry weight was one of the highest levels ever reported [8]. Another alternative solution to avoid substrate limitation for PC production was demonstrated by Singh *et al.* [9] by expressing PC synthase in *S. cerevisiae*, which has a sufficient supply of GSH. Since the incorporation of sulfide into PC–metal complexes has been shown to be responsible for detoxification in yeast [10], it may be possible to further enhance the overall heavy metal accumulation by aerobic production of H_2S [11].

One of the limitations with MTs or PCs is their non-selective binding to a variety of heavy metals. Specific heavy metal transporters, in addition to their ability to enhance uptake, have been employed to enhance the specific accumulation of target heavy metals. Selective Cd accumulation was demonstrated by Kim *et al.* [12] by expressing the Cd transporter MntA and the yeast MT. Another successful approach is to harness naturally evolved metal-binding MTs from specific metal contamination sites. Recently, Singh *et al.* [13•] reported the utility of a fMT from an arsenic-tolerant marine alga, *Fucus vesiculosus* [14]. Co-expression of fMT with a specific arsenic transporter GlpF in *E. coli* results in 45-fold increase in arsenic accumulation. More importantly, even resting cells can be used to completely remove 35 ppb of As(III) within 20 min, making this a low-cost option for arsenic removal. It is possible that similar approaches can be used for other heavy metal contaminants.

Many microorganisms have developed natural resistant pathways for heavy metals, which are tightly controlled by a specific metalloregulatory protein. The high affinity and selective nature of these metalloregulatory proteins have been exploited for the specific accumulation of mercury and arsenic [15•,16]. Even a minimal MerR domain consisted of a single polypeptide ordinarily formed between two monomers of MerR was designed with high affinity to bind mercury [17]. Other enzymes involved in the resistant pathway such as the mercuric reductase (merA), have

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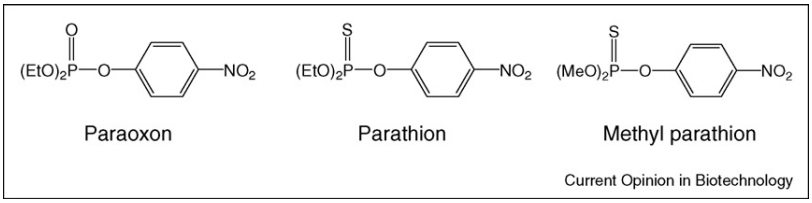
Figure 1



Examples of organophosphates.

also been used in conjunction with polyphosphate, a phosphate polymer, for removal of inorganic and organic mercury [18].

In addition to heavy metals, radionuclide contamination either through nuclear plant leaks or by nuclear weapons is a major environmental issue [19]. Naturally occurring bacteria highly resistant to radiation are ideal metabolic engineering candidates for enhanced radionuclide clean-up. Brim *et al.* [20••] first reported the use of an extremely radiation-resistant and thermophilic bacterium *Deinococcus geothemalis* by expressing the *mer* operon from *E. coli* coding for Hg^{2+} reduction. The engineered bacteria were capable of reducing mercury at higher temperature and ionizing radiation. The engineered bacteria also had the capability to reduce Fe(III), U(VI), and Cr(VI). This demonstrates the possibility of utilizing such engineered microorganisms for mixed radioactive wastes and at higher temperatures. Polyphosphates could also be used in *P. aeruginosa* for radionuclide precipitation as metal phosphate by overexpressing polyphosphate kinase and exopolyphosphatases [21]. Recently a non-specific phosphatase phoN was expressed in a radiation-resistant bacterium *D. radiodurans* leading to bioprecipitation of uranium from dilute nuclear waste [22•]. Directed evolution has also been used to improve the enzymatic efficiency of chromate and uranyl reductases; engineered enzymes expressed in *E. coli* and *P. putida* showed improved enzymatic kinetics [23•]. It is clear that these improved enzymes when expressed in radiation-resistant microorganism could further enhance the radionuclide precipitation efficiency.

Engineered microbes for organic chemical removal

Bioremediation of organic pollutants involves improved degradation of these chemicals and their transformation into less toxic or non-toxic products. Microorganisms have been evolved to degrade and utilize many of these organic chemicals; however, these degradation pathways are limited by the relative slow rate. In the following section we will discuss about recent developments to enhance bioremediation of common organic pollutants through pathway engineering of various microorganisms.

The widespread use of organophosphates (OPs) in agriculture as pesticides has led to serious environmental pollution by this extremely toxic compound. OPs are the ester forms of phosphoric acid and most widely used insecticides including paraoxon, parathion, or methyl parathion (Figure 1). Naturally occurring soil bacteria have evolved the ability to degrade OPs with the help of an enzyme called organophosphate hydrolase (OPH or phosphotriesterase) [24,25]. OPH catalyzes the hydrolysis of P-O linkage releasing *p*-nitrophenol as a leaving group. Since the toxicity of OPs is significantly reduced by hydrolysis of phosphoester bonds, many researchers have focused on the initial hydrolysis by OPH [26].

Genetic engineering techniques such as DNA shuffling, site-directed mutagenesis, or error-prone PCR have been applied to improve the catalytic activity and substrate specificity of OPH [27–30]. Mutants with 25-fold and 725-fold higher hydrolytic activity for two poorly hydrolysable OPs, methyl parathion, and chlorpyrifos, respectively, were obtained using DNA shuffling [27,30]. In addition, translocation of the enzyme to the cell surface was used to enhance biodetoxification efficiency by eliminating the transport bottleneck of OPs across the cell membrane. OPH was displayed on the surface of *E. coli* using the Lpp (lipoprotein)–OmpA (outer membrane protein A) system and led to 7-fold faster degradation of parathion than intracellularly expressed OPH [31]. Recently, efforts were made by exploiting the sec pathway for OPH secretion to the periplasmic space in order to improve the overall hydrolysis activity [32].

Although some OPs are transformed by the initial hydrolysis to much less toxic *p*-nitrophenol (PNP) and dialkyl phosphates, these degraded products still are toxic and resistant to biodegradation [33,34]. One approach is to exploit a microbial consortium with the required degradation pathways. A co-culture of engineered *E. coli* with ability to hydrolyze parathion and diethylthiophosphate and the natural PNP-degrader *Pseudomonas putida* was used to successfully decompose parathion without accumulation of PNP [35]. A less complicated strategy utilizing either engineered *Moraxella* sp. or *P. putida* that can naturally degrade PNP has been reported. In the

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former, OPH was expressed on the cell surface and demonstrated to simultaneously degrade parathion, methyl parathion and paraoxon, and their hydrolysis product PNP [36]. In the later, a *P. putida* strain, containing a natural PNP degradation operon was used. A synthetic operon for expression of OPH, phosphodiesterase (Pde), and alkaline phosphatase (PhoA), was introduced to enable the hydrolysis of OPs and diethyl phosphate (DEP) mineralization. The resulting *P. putida* strain was able to completely degrade paraoxon, PNP, and DEP within 24 h, 78 h and 142 h, respectively. Even with the growth inhibition by PNP and the low phosphate availability caused by inefficient degradation of DEP, this work presents a potentially novel approach to create an artificial metabolic pathway for complete mineralization [37••].

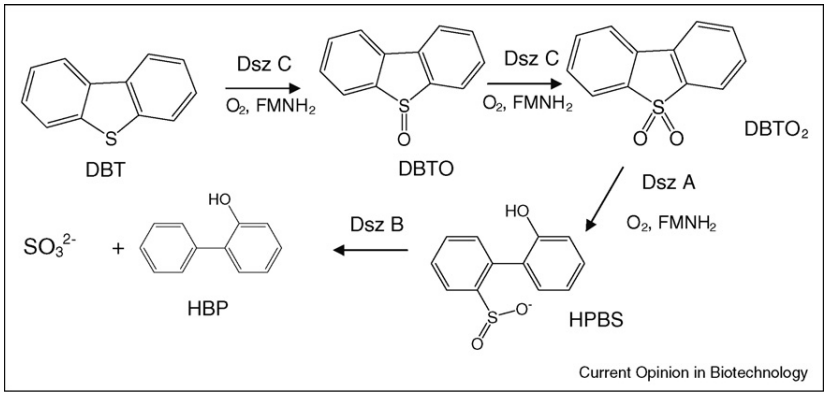
The current hydrodesulfurization method for lowering the level of organic sulfur compounds is unable to satisfy the stringent government regulations. Current advances in desulfurization have therefore been focused on biological processes using microorganisms. Among the organosulfur compounds, dibenzothiophene (DBT) is a popular model molecule because the hydrodesulfurization method is not effecient to remove DBT from fossil fuels [38]. The desulfurization for DBT requires a series of enzymes (Figure 2). The first enzyme is dibenzothiophene monooxygenase (DszC) that converts DBT to DBT sulfone ($DBTO_2$). The second enzyme dibenzothiophene-5,5-dioxide monooxygenase (DszA) is responsible for the conversion of $DBTO_2$ into 2-hydroxybiphenyl-2-sulfinate (HBPS), and 2-hydroxybiphenyl-2-sulfinate sulfinolyase (DszB) catalyzes the conversion of HBPS into 2-hydroxybiphenyl (HBP) and sulfite [39].

In the desulfurization pathway, the last step is known as the rate limiting reaction [40]. Overexpression of DszB was achieved by mutation at the 5′ untranslated region of dszB. The resulting mutant improved desulfurization rate by 9-fold compared with cells expressing the native dszB [39]. Another study regarding improvement of DszB was reported by Li *et al.* [41]. Expressional characterization of the native dsz operon showed that the mRNA ratio of dszA, dszB, and dszC was 11:3.3:1, but the enzyme level of DszB was the lowest. This low translation rate for dszB is a result of an overlap between the dszA stop codon and the dszB start codon. For stable expression of DszB, this enzyme was redesigned with inserted sequences containing a potential ribosome binding site, and cells expressing the reconstructed operon exhibited 5-fold higher desulfurization than that of native operon [41].

Evolution of DszB was also attempted to enhance the enzyme activity [42]. Site-directed mutagenesis based on structure information improved DszB activity and thermostability. Rather than focusing on only a single step, the order of genes on the operon was rearranged to increase desulfurization and to better coordinate the enzyme activities [43••]. By changing the ratio of dszA, dszB, and dszC mRNAs to 1:16:5, the overall desulfurization activity was improved by 12-fold. This rearrangement strategy suggests the successful application of a rational approach to harmonize the whole metabolic pathway.

Nitroaromatic compounds such as nitrobenzene or nitrotoluene are widely used as pesticides, dyes, polymers, or explosives and are considered as priority pollutants [44]. Most microorganisms transform nitroaromatic compounds into amines by redox enzymes. However, the biodegradation rate is very slow owing to the toxicity and the electron-withdrawing effect of nitro groups. Although bacteria strains that mineralize nitrobenzene, nitrotoluene, and dinitrotoluene were isolated and the enzymes

Figure 2



The metabolic pathway for DBT desulfurization. DBT is desulfurized to HBP via two monooxygenases (DszC and DszA) and a desulfinase (DszB). This pathway requires the oxidative condition with the help of reduced flavin mononucleotide (FMNH2). The intermediates are dibenzothiophene-5-oxide (DBTO); dibenzothiophene-5,5-dioxide ($DBTO_2$); 2-(2-hydroxybiphenyl)-benzenesulfinase (HBS or HBPS) (adapted from reference [39]).

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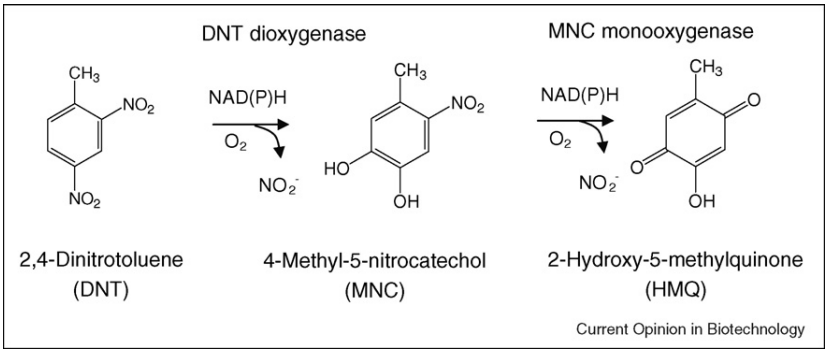
Figure 3



The oxidative pathway for DNT degradation. The conversion of DNT into HMQ is catalyzed by two oxygenases (DNT dioxygenase and MNC monooxygenase) releasing nitrite (adapted from reference [53]).

involved in the pathway for biodegradation were elucidated, the lack of specificity of these enzymes causes natural degradation to occur at a very slow pace [45–47].

Rational design of enzymatic activity has been used to improve the degradation of nitroaromatic compounds. Nitrobenzene 1,2-dioxygenase catalyzes the conversion of nitrobenzene to catechol and nitrite [45]. The residues near the active site of this enzyme were modified for controlling substrate specificity. The substitution of amino acid at the position 293 (F293Q) expanded substrate specificity, resulting in 2.5-fold faster oxidization rate against 2,6 dinitrotoluene [48]. The same approach was taken to 2-nitrotoluene dioxygenase that is responsible for the oxidation of nitrotoluene to 3-methyl catechol and nitrite [46]. The residues near active sites were chosen for site-directed mutagenesis and the replacement at the position 258 (N258V) significantly changed the enantiospecificity [49].

2,4–dinitrotoluene dioxygenase (DNTDO) is another enzyme that catabolizes 2,4-dinitrotoluene into 4-methyl-5-nitrocatechol and nitrite [47]. This enzyme is a multi-component enzyme system consisting of flavonprotein reductase, iron–sulfur ferredoxin, and α and β subunits [50]. On the basis of the importance of the A206 position of a similar naphthalene dioxygenase on regioselectivity [51], the analogous position I204 of DNTDO was mutated to broaden the enzyme specificity for substrates [52]. Significant improvements for various dinitrotoluenes (DNTs) such as 2,3-DNT, 2,5-DNT, 2,6-DNT, 2NT, and 4NT were obtained by saturation mutagenesis. A mutant I204L was reported to degrade 2,3-DNT and 2,5-DNT that could not be transformed by the wild-type enzyme. This mutant also oxidized 2,4-DNT and 2,6-DNT 2-fold faster and exhibited higher activity on 2-NT and 4-NT by 3.5-fold and 8-fold, respectively. This group subsequently reported the engineering of a second enzyme in the DNT catabolic pathway [54•]. 4-Methyl-5-nitrocatechol (MNC), the initial product of DNT degradation, is converted to 2-hydroxy-5-methylquinone by the MNC monooxygenase (Figure 3) [53]. Since there is no crystal structure available, error-prone PCR methods were used [54•]. MNC monooxygenase has a very narrow substrate specificity accepting only MNC and 4-nitrocatechol. [53]. However, a new mutant with changes in two residues (M22L/L380I) could transform 4-nitrophenol and 3-methyl-4-nitrophenol and showed 11-fold higher efficiency than wild type enzyme for 4-nitrophenol degradation. Although the M22L/L380I mutant increased the initial rate for MNC degradation less than 2-fold, the combination of this mutated enzyme with I204L enzyme at the first step can be a synergic approach, eliminating the probable inhibition of the DNT dioxygenase activity by MNC.

Irrational approaches such as genome shuffling have also been explored for the biodegradation of another important pesticide pentachlorophenol (PCP) [55•]. Three rounds of genome shuffling of PCP-degrading *Sphingobium chlorophenolicum* resulted in strains with enhanced growth, improved PCP degradation, and resistance to PCP toxicity [55•]. Some mutant strains were able to completely degrade PCP. Furthermore, genome-wide approaches like global functional genomics, physiological analysis, and modeling could lead to enhanced microorganisms with optimized biodegradative pathways at multiple levels [56].

New toxic-chemical degrading enzymes and pathways are currently enthusiastically sought after. Cytochrome P450 has been extensively studied and various protein engineering techniques have been applied for improved biodegradation functions. However, recently an unusual explosive degrading P450 system able to degrade a highly toxic explosive hexa-hydro-1,3,5-trinitro-1,3,5-triazine

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(RDX) was characterized [57]. The newly discovered XplA and XplB, when overexpressed in *Arabidopsis*, yielded very rapid RDX removal. In future, these systems could be transferred to microbial machinery for faster and rapid explosive removal. Another interesting approach is to exploit the specialized membrane structure 'superchannels' from *Sphingomonas* sp. A1 for enhanced pollutant uptake [58•]. Introduction of these superchannels in dioxin-degrading *S. wittichii* RW1 and the polypropylene glycol degrading *S. subarctica* IFO 16058T resulted in substantial enhancement in bioremediation capacity. This could be a general approach and be applied to other engineered biodegrading microorganisms; however, energy dependent transport through these superchannels might limit their applicability.

Conclusion

Various molecular, genetic, and metabolic engineering tools have accelerated the progress toward bioremediation and have led to specifically designed microorganism for various bio-based clean-up processes. The ever expanding knowledge base about biodegradation pathways, structural and functional knowledge of key enzymes, and extensive molecular characterization of naturally detoxifying microorganisms will further our progress to cleverly designed pathways toward a new era of bioremediation.

Acknowledgements

The financial supports from National Science Foundation (BES0422791 and BES0329482) and Environmental Protection Agency (R833008) are gratefully acknowledged.

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