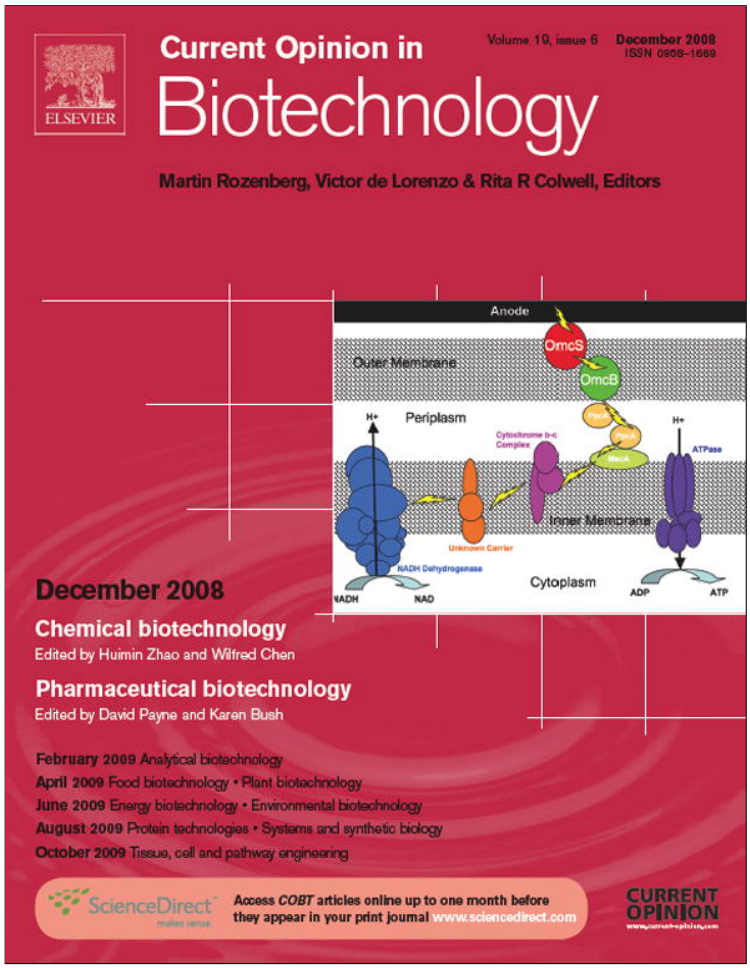

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Molecular approaches in bioremediation

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Bacteria have enormous catabolic potential for remediating wastes; however, the interactions between bacteria and pollutants are complex and suitable remediation does not always take place. Hence, molecular approaches are being applied to enhance bioremediation. Here, an overview is provided of the recent advances in bioremediation by utilizing rhizoremediation, protein engineering, metabolic engineering, whole-transcriptome profiling, and proteomics for the degradation of recalcitrant pollutants such as chlorinated aliphatics and polychlorinated biphenyls as well as for binding heavy metals.

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Introduction

Bioremediation

Bioremediation is the degradation of pollutants using organisms, from bacteria to plants, or their derivatives. The chief advantage of bioremediation is its reduced cost compared to conventional techniques such as incineration since the cost of remediation for all contaminated sites in the USA alone is estimated to be $1.7 trillion [1]. In addition, bioremediation is often a permanent solution (providing complete transformation of the pollutant to its molecular constituents like carbon dioxide and water) rather than a remediation method that transfers wastes from one phase to another [1]. Unfortunately, there are many man-made compounds that lack good biological catalysts (there are 10 million described organic compounds and biodegradation for most of them has not been investigated [2]), and many instances where good biocatalysts fail to transform pollutants in the environment [3].

One advance in bioremediation to improve the stability of the biocatalyst is to create a system where degradation occurs in the area near the roots of plants known as the rhizosphere; the term rhizoremediation was coined in 1998 in a study of the degradation trichloroethylene (TCE) in the wheat rhizosphere by bacteria expressing a stable, chromosomally encoded toluene *ortho*-monooxygenase (TOM) (Figure 1) [4••]. In rhizoremediation, the bacteria degrade the pollutants while the plant roots provide a niche for the microorganism and key nutrients. The advantages of rhizoremediation include that the plant roots provide a large surface area for bacterial propagation and biofilm formation, that the roots transport the bacteria through the contaminated soil, that the roots provide a niche for the bacteria by providing nutrients, and that the roots facilitate oxygen exchange [3]. The requirements for successful rhizoremediation include that the bacteria adhere well to seeds, that the bacteria proliferate well in the root system, and that the degradative pathways operate well in the rhizosphere [3]. Successful rhizoremediation systems have been established for pollutants such as chlorinated ethenes, polycyclic aromatic hydrocarbons, polychlorinated biphenyls (PCBs), fuels, metals, and parathion [1]. This review explores the use of molecular approaches designed to overcome obstacles to successful bioremediation and focuses on rhizoremediation as a bioremediation method that is gaining importance and on the degradation of chlorinated ethenes since they are among the most-frequently encountered groundwater pollutants [5].

Review

Protein engineering

Directed evolution or DNA shuffling [6,7,8,9••] is a powerful mutagenesis technique that mimics the natural molecular evolution of genes in order to efficiently redesign them. Its power lies in that it can introduce *multiple* mutations into a gene in order to create new enzymatic activity (found by a suitable method of selection); it is still difficult (if not impossible) to *rationally* predict the amino acid changes that occur during DNA shuffling and that are necessary to create the new activity. These mutations are found both near and far from the active site [10]. This method was developed by Willem Stemmer of Affymax Research Institute (now Maxygen) and consists of using PCR without oligo primers to re-assemble a gene (or a family of genes) from random 10 to 300 bp DNA fragments generated by first cleaving the gene with DNase. After re-assembling the original gene from these 300 bp

Figure 1

Rhizoremediation of trichloroethylene in a microcosm: root-colonizing *Pseudomonas fluorescens* was engineered to express toluene *o*-monooxygenase of *Burkholderia cepacia* G4 from its chromosome [4••]. The resulting strain degraded 64% of the added trichloroethylene as it grew on wheat roots.

fragments using a series of homologous recombinations and extensions with dNTPs and polymerase, normal PCR (with nested oligos) is performed using traditional oligos to yield the full-length gene with random mutations. The mutations arise from infidelity in the assembly process, PCR infidelity (polymerase base-reading errors), and errors introduced in the assembly process by insertion of mutated gene fragments (controlled by the researcher by adding specific oligos or DNA fragments from related but not identical genes). The advantages of this method are that DNA shuffling introduces mutations much more efficiently than other methods (e.g., unlike DNA shuffling, error-prone PCR and oligonucleotide cassette mutagenesis are not combinatorial), and it may be used to create a chimeric gene by reassembling closely related genes (family shuffling). This method has been used to increase β-lactamase antibiotic activity by 32 000-fold [9••], to increase the fluorescence signal of the green fluorescent protein by 45-fold [7], and to evolve a fucosidase from β-galactosidase [11].

After random protein engineering, saturation mutagenesis is extremely powerful in creating new catalysts for bioremediation as it can be used to introduce all possible mutations at key sites or adjacent sites to explore a larger fraction of the protein sequence space that can be achieved with site-directed mutagenesis [12]. It can provide much more comprehensive information than can be achieved by single-amino acid substitutions as well as overcome the drawbacks of random mutagenesis in that a single mutation randomly placed in codons generates on average only 5.6 out of 19 possible substitutions [13]. To use saturation mutagenesis effectively, it is necessary to determine the number of independent colonies that must be screened to ensure that each possible codon has been tested; hence, a multinomial distribution equation was developed to predict the number of colonies required as a function of the number of sites mutated assuming that 64 codons are randomized at each position [14••]. For example, for saturation mutagenesis of one site, 292 colonies need to be screened for a probability of 0.99 that all 64 codons are sampled, and if two residues are subject to simultaneous saturation mutagenesis, 342 independent clones need to be sampled to ensure the 0.99 probability that all the possible outcomes have been checked [14••]. Like DNA shuffling, saturation mutagenesis requires a suitable selection or screening method [15].

DNA shuffling has been used successfully to create a biocatalyst with higher degradation rates for chlorinated ethenes (trichloroethylene, 1,1-dichlorethylene, and trans-dichloroethylene) and polyaromatic hydrocarbons (naphthalene, phenanthrene, fluorene, and anthracene) [16•]. Random mutagenesis over 3.5 kb that included five of the six genes that encode TOM of *Burkholderia cepacia* G4 led to the discovery of an important gate residue in the large subunit of the hydroxylase, valine 106 [16•]. The variant with the V106A mutation was called TOM-Green [16•] since it caused the complex fermentation broth to turn green owing to the formation of colored indigoid compounds that form owing to oxidation of indole by TOM-Green [17]. Saturation mutagenesis was then utilized at position V106 to increase the activity of this enzyme for chloroform [14••]. A combination of DNA shuffling and saturation mutagenesis was also used to evolve another monooxygenase, toluene-*o*-xylene monooxygenase from *P. stutzeri* OX1 (renamed *Pseudomonas* sp. OX1 [18]) for enhanced chlorinated ethene degradation [19] and to discover new residues for accelerating *p*-nitrophenol degradation [10]. The techniques of DNA shuffling and saturation mutagenesis have also been utilized to evolve dioxygenases and monooxygenases for the bioremediation of nitroaromatics such as dinitrotoluenes (including 2,3-dinitrotoluene and 2,5-dinitrotoluene that previously could not be degraded [20•]), 4-methyl-5-nitrocatechol (one of the first examples of the evolution of an enzyme for the second step of a biodegradative pathway [21]), aminonitrotoluenes [22•], and nitro-phenols/methoxy-phenols [23]. The insights gained from these biodegradation studies with oxygenases are important also for biocatalysis and green chemistry; for example, by discovering important residues in the active site of toluene monooxygenases, it is now possible to control regio-specific oxidation toluene [24••].

In one of the more successful uses of protein engineering for bioremediation, bacteria were created that utilize 1,2,3-trichloropropane (TCP) as a carbon and energy source [25••]. After trying unsuccessfully to use bacterial enrichment cultures to find a TCP-utilizing strain, the authors evolved haloalkane dehalogenase from *Rhodococcus* sp. m15-3 to allow more productive binding of TCP in the active site then added this improved enzyme to *Agrobacterium radiobacter* AD1 that utilizes the product of the haloalkane dehalogenase reaction.

One of the more clever screening schemes was devised for evolving organophosphorus hydrolase for organophosphorus pesticide degradation; the enzyme was displayed on the exterior of *Escherichia coli* using a truncated ice nucleation protein (so mass transfer limitations are removed) and active colonies were isolated on the basis of the formation of yellow *p*-nitrophenol from methyl parathion [26••]. After two rounds of DNA shuffling, an organophosphorus hydrolase variant was identified with 25-fold greater degradation of the pesticide methyl parathion.

Family and genome shuffling for PCBs and pentachlorophenol

Family shuffling applies DNA shuffling to groups of related genes to combine them in a manner that accelerates directed evolution [27]. One of the first applications of protein engineering for bioremediation was the family shuffling evolution of the large subunit of biphenyl dioxygenase (*bphA*) from *P. pseudoalcaligenes* KF707 and *B. cepacia* LB400 [28•]; the hybrid enzymes had enhanced degradation of PCBs, biphenyl compounds, toluene, and benzene. This method has also been applied for the degradation of PCBs by shuffling crucial segments of *bphA* genes from *Burkholderia* sp. strain LB400, *Comamonas testosteroni* B-356, and *Rhodococcus globerulus* P6 [29]. Biphenyl dioxygenase variants were identified with activity to a broader range of PCBs than the parent enzymes.

Genome shuffling recombines the chromosomes of several bacteria to improve activity of the whole organism [30]. This method has been applied to bioremediation for the degradation of pentachlorophenol to create strains that could grow in 10-fold higher concentrations of pentachlorophenol and that degrade completely concentrations that could not be used by the wild-type strain [31•]. This work is also interesting since it applied genome shuffling to a Gram-negative strain.

Metabolic engineering for chlorinated aliphatics

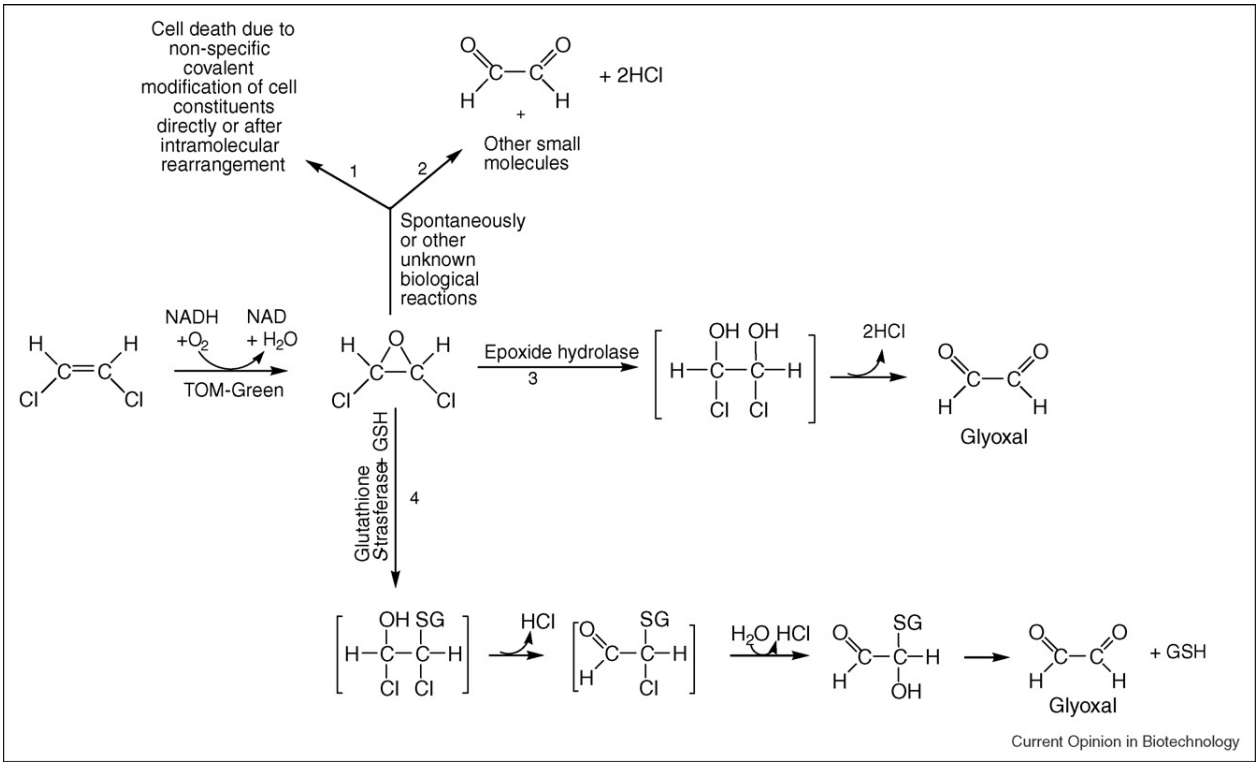
Beyond optimizing a single enzyme, metabolic engineering involves redirecting the cell's metabolism to achieve a particular goal using recombinant engineering [32]. One of the first and finest examples of this approach is the metabolic engineering of *Pseudomonas* sp. B13; five different catabolic pathways from three different bacteria were combined to allow for degradation of methylphenols and methylbenzoates in a single organism [33••].

Metabolic engineering has also been used to create strains that degrade chlorinated ethenes more readily through the use of several cloned enzymes. The rationale for this metabolic engineering is that the Gibbs free energy change for aerobic degradation of chlorinated ethenes to water, carbon dioxide, and HCl, indicates growth on nearly all chlorinated aliphatics is thermodynamically possible; for example, the Gibbs free energy change for the aerobic mineralization of *cis*-1,2-dichloroethylene, *cis*-DCE, is −1143 kJ/mol [34] and even fully chlorinated ethane may be degraded [35•]. If the reactive intermediates may be effectively detoxified, the main biochemical factor that hampers chlorinated ethenes from supporting cell growth is the lack of appropriate enzymes to harvest their energy. Hence it is feasible to construct bacteria that grow on these chlorinated ethenes and create a niche for their aerobic degradation. Currently, the aerobic degradation of chlorinated ethenes is fortuitous and provides no benefit to the cell; in fact, it leads to cell death and thus is selected against (Figure 2).

The toxic epoxides generated during the aerobic biodegradation of chlorinated ethenes limit their transformation. Hydrolysis of the toxic epoxide by epoxide hydrolases represents the major biological detoxification strategy; however, chlorinated epoxyethanes are not accepted by known bacterial epoxide hydrolases. The epoxide hydrolase from *A. radiobacter* AD1 (EchA), which enables growth on epichlorohydrin, was therefore evolved to accept *cis*-1,2-dichloroepoxyethane as a substrate by accumulating beneficial mutations from three rounds of saturation mutagenesis at three selected active site residues: F108, I219, and C248 [36••]. The EchA F108L/I219L/C248I variant co-expressed with DNA-shuffled TOM (TOM-Green), which initiates attack on the chlorinated ethene, allowed for the degradation of *cis*-DCE at low concentrations (6.8 μM) (wild-type EchA has no activity at this concentration) and enhanced degradation 10-fold at high concentrations (540 μM) (Figure 2). For complete degradation of *cis*-DCE to chloride ions, the apparent V_{max}/K_m for the recombinant *E. coli* strain expressing the EchA F108L/I219L/C248I variant was increased over five-fold as a result of the evolution of EchA; hence, the addition of seven foreign genes led to greater degradation of chlorinated aliphatics.

A similar metabolic engineering approach for degrading chlorinated ethenes using a glutathione *S*-transferase (GST) and an overexpressed *E. coli* mutant γ-glutamylcysteine synthetase (GSHI*) instead of the engineered epoxide hydrolase has also been implemented (Figure 2) [37]. A recombinant *E. coli* strain less sensitive to the toxic effects of *cis*-DCE, TCE, and *trans*-1,2-dichloroethylene

Figure 2



Metabolic engineering to enhance *cis*-dichloroethylene (*cis*-DCE) mineralization by cloning an evolved toluene *o*-monooxygenase (TOM-Green) to initiate oxidation of *cis*-DCE with either (i) an evolved epoxide hydrolase (EchA) that adds water to the epoxide bond to detoxify it [36••] or (ii) glutathione *S*-transferase (IsoILR1) along with an overexpressed *E. coli* mutant γ-glutamylcysteine synthetase (GSHI*) [37]; GSHI* overproduces glutathione that is added to the epoxide bond by IsoILR1. Steps 1 and 2 are the two possible spontaneous transformation pathways for *cis*-DCE epoxide, while step 3 and step 4 represent two major detoxification strategies in which *cis*-DCE epoxide may be biologically converted by either an epoxide hydrolase (EchA) or glutathione *S*-transferase (IsoILR1). Figure used with permission from ASBMB [36].

(*trans*-DCE) degradation was created by engineering a novel pathway consisting of eight genes including DNA-shuffled TOM-Green, a newly discovered glutathione *S*-transferase (GST) from *Rhodococcus* AD45 (IsoILR1) found to have activity toward epoxypropane and *cis*-DCE epoxide, and an *E. coli* mutant γ-glutamylcysteine synthetase (GSHI*) that is not subject to feedback inhibition. The recombinant strain in which TOM-Green and IsoILR1 were co-expressed on separate plasmids degraded 1.9-fold more *cis*-DCE compared to a strain that lacked IsoILR1. In the presence of IsoILR1 and TOM-Green, the addition of GSH1* resulted in a 7-fold increase in the intracellular GSH concentration and a 3.5-fold improvement in the *cis*-DCE degradation rate based on chloride release, a 1.8-fold improvement in the *trans*-DCE degradation, and a 1.7-fold improvement in the TCE degradation rate. In addition, cells expressing IsoILR1 and GSHI* grew 78% faster in rich medium than a strain lacking these two heterologous genes [37]. Therefore, this method was successful in reducing the toxicity related to aerobic degradation of chlorinated aliphatics.

Metabolic engineering for mixed wastes

Metabolic engineering has also been used successfully to handle mixtures of pollutants. For example, many superfund sites are contaminated with both chlorinated ethenes such as TCE and heavy metals; hence, a promising strategy to address these mixed-waste situations is the use of TCE-degrading rhizobacteria that will thrive in soil heavily polluted with heavy metals. To achieve this goal, the metal-binding peptide, EC20, a synthetic phytochelatin with general structure $(Glu\text{-}Cys)_{20}$, was expressed on the cell surface of rhizobacteria engineered to degrade TCE (strain Pb2-1 and *Rhizobium* strain 10320D [38]), resulting in strains that both accumulated metal and degraded TCE [39]. EC20 was displayed on the cell surface of *Pseudomonas* using an ice-nucleation protein anchor and resulted in 6-fold higher cadmium accumulation. As expected, the TCE degradation rate was reduced in the presence of cadmium for cells without EC20 expression; however, expression of EC20 (higher cadmium accumulation) completely restored TCE degradation. These results demonstrated that EC20 expression enhanced not only cadmium accumulation but also

reduced the toxic effect of cadmium on TCE degradation for the bacterium. Expressing EC20 on another rhizobacterium, *P. putida* 06909, has also been shown to reduce cadmium toxicity to sunflowers and to increase cadmium accumulation [40].

Whole-transcriptome profiling to facilitate rhizoremediation

Whole-transcriptome profiling using DNA microarrays has the advantage that the relative amount of transcripts from the whole genome may be easily determined compared to such techniques like proteomics that, in general, are not able to discern the identity readily for all proteins (e.g., membrane-bound proteins are problematic and roughly 2/3 of *E. coli* proteins have not been identified by non-gel proteomic techniques [41]). However, transcriptome profiling often assumes that changes in transcription may be used to predict changes in protein formation that may not always be correct but is often true for procaryotes since regulation occurs primarily at the level of transcription.

To understand the metabolism of bacteria in the rhizosphere, several groups have begun to utilize whole-genome profiling. The first whole-transcriptome study of bacteria and plants was that of *Erwinia chrysanthemi* on African violet leaves in which several virulence genes were identified [42]. The first whole-transcriptome study of bacteria in the rhizosphere was conducted with poplar tree roots and pathogenic *Pseudomonas aeruginosa* in which seven novel bacterial virulence genes were identified [43••]; note that this manuscript also investigated the whole-transcriptome response of the plant to the pathogenic bacterium. For bioremediation, whole-transcriptome profiling has been used to determine mutualistic interactions in the rhizosphere for strains relevant for bioremediation; for example, 90 rhizosphere upregulated genes were identified for *P. putida* growing on corn roots [44••].

Bacteria have also been selected to perform well in rhizoremediation; for example, to facilitate the degradation of the pesticide lindane (γ-hexachlorocyclohexane) as well as the related compounds δ-hexachlorocyclohexane and β-hexachlorocyclohexane, Ramos and co-workers utilized a double enrichment approach to isolate four *Sphingomonas* strains that degrade lindane and that proliferate in the corn rhizosphere whereas the parent strains could not colonize the plant [45]. This approach holds promise for combining bacteria with strong degradation potential with suitable plant hosts.

Proteomics in bioremediation

Although whole-transcriptome approaches are important, this approach is unable to show that changes in transcription lead to changes in protein levels and frequently changes in proteins are necessary for enhanced bioremediation. Hence, one of the best techniques for gauging changes in metabolism is proteomics, that is, determining the complete change in protein production in the cell.

One of the first attempts to gauge the impact of metabolic engineering in bioremediation was a proteomics study of TCE degradation in which two dimensional electrophoresis (2-DE) was used to detect changes in the proteome of *E. coli* cells upon expressing toluene *o*-monooxygenase (TOM) that converts TCE into an reactive TEC epoxide; eight new proteins were identified in TOM-containing cells and 12 proteins not detected in those cells were present in the host strain [46]. Exposure of TOM-containing cells to TCE led to the synthesis of only one new protein and the loss of 10 proteins. Therefore, metabolic engineering (addition of the TOM enzyme) has a substantial and complex impact on the physiology of these cells that was clearly revealed using a proteomic approach.

A more thorough proteome investigation [48••] was conducted on the basis of the metabolic engineering of *E. coli* to degrade *cis*-1,2-dichloroethylene (*cis*-DCE) as shown in Figure 2 and described above. The strains express the six genes of an evolved toluene *o*-monooxygenase (TOM-Green, which form a reactive epoxide) with either (1) γ-glutamylcysteine synthetase (GSHI*, which forms glutathione) and the glutathione *S*-transferase IsoILR1 from *Rhodococcus* AD45 (which adds glutathione to the reactive *cis*-DCE epoxide) or (2) with an evolved epoxide hydrolase from *A. radiobacter* AD1 (EchA F108L/I219L/C248I that converts the reactive *cis*-DCE epoxide to a diol). The impact of this metabolic engineering for bioremediation was assessed by investigating the changes in the proteome through a quantitative shotgun proteomics technique (iTRAQ) by tracking the changes due to the sequential addition of TOM-Green, IsoILR1, and GSHI* and due to adding the evolved EchA vs. the wild-type enzyme to TOM-Green. For the TOM-Green/EchA system, nine proteins out of 268 identified proteins were differentially expressed in the strain expressing EchA F108L/I219L/C248I relative to wild-type EchA. For the TOM-Green/IsoILR1/GSHI* system, the expression level of 49 proteins was changed out of 364 identified proteins. It was determined that the metabolic engineering that leads to enhanced aerobic degradation of *cis*-DCE and reduced toxicity from *cis*-DCE epoxide results in enhanced synthesis of glutathione coupled with an induced stress response as well as repression of fatty acid synthesis, gluconeogenesis, and the tricarboxylic acid cycle.

Concluding remarks

The success of bioremediation involves complex interactions and so it is envisioned that sophisticated metabolic techniques will continued to be implemented to advance the field. Although a tremendous amount of work

remains to be performed, significant advances have been made through protein engineering and through metabolic engineering for bioremediation. However, as shown by this brief review, even though whole-transcriptome profiling and proteomics are utilized routinely in many disciplines, they remain to be utilized extensively in bioremediation. Furthermore, it is important to ensure engineered strains designed for field use are competitive; chromosomal integration of genes has been shown to be effective in this regard [38], and rhizoremediation provides a niche for these engineered bacteria. Chromosomal integration also limits horizontal gene transfer [47] but this should also be verified.

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