

Although we do not yet fully understand how the cell produces siRNAs and uses them to cleave complementary targets, we do know that organisms have been using such mechanisms for eons for essentially these purposes. For this reason, siRNAs could be the most versatile and widely applicable new technology for affecting cellular processes at the level of gene expression.

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'Super bugs' for bioremediation

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Chlorinated organic compounds are among the most significant pollutants in the world. Sequential use of anaerobic halo-respiring bacteria, which are the key players in biological dehalogenation processes, and aerobic bacteria whose oxygenases are modified by directed evolution could lead to efficient and total degradation of highly chlorinated organic pollutants. Recently three interesting papers on halo-respiration and polychlorinated biphenyl biodegradation were published.

There is great concern over chlorinated organic compounds because of their toxicity, persistence and bioaccumulation. Among these compounds, polychlorinated biphenyls (PCBs) and chlorinated organic solvents such as trichloroethene (TCE), tetrachloroethene (PCE) and 1,1,1-trichloroethane (TCA) are the major targets for bioremediation. TCE, PCE and TCA were widely used and are recognized as serious environmental contaminants in soil, groundwater and the atmosphere. An increasing number of bacteria has been isolated that can couple reductive dehalogenation of these chlorinated solvents with energy conservation [1]. A halo-respiratory process would therefore be

effective for *in situ* bioremediation of these chlorinated solvents. Microbial degradation of PCBs has been extensively documented in terms of the biodegradability and molecular characteristics of enzymes and genes from a variety of soil bacteria [2]. Because PCBs are complicated mixtures containing up to ten chlorine atoms on a biphenyl molecule, microbial degradation is highly dependent on chlorine substitution and is highly strain dependent. Recently, attempts have been made to enhance PCB biodegradation by modifying oxygenases [3]. One of the most efficient methods of biological degradation consists of sequential anaerobic–aerobic treatment for highly chlorinated compounds. Recent biochemical and genetic engineering approaches for dehalogenases and oxygenases could lead to 'super bugs' that could be used for the bioremediation of chlorinated environmental pollutants.

Microbial halo-respiration

An increasing number of bacteria has been isolated that can couple the reductive dehalogenation of various chlorinated compounds to energy conservation by electron-transport-coupled phosphorylation [1]. This process is referred to as halo-respiration, or dehalorespiration. Recent studies indicate that halo-respiring bacteria have

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a crucial role in the degradation of highly chlorinated hydrocarbons such as chlorobenzenes and PCE and these organisms are considered important for the effective removal of chlorinated pollutants from contaminated environments. Their dehalogenation activities are several orders of magnitude higher than those observed for co-metabolic processes.

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In the case of PCE, several strict anaerobes have been isolated and characterized [1,4]. Molecular characterization of reductive dehalogenases from these organisms revealed that the enzymes constitute a novel class of reductases containing complex tetrapyrrol cofactors (corrinoid and heme) [5–7]. All the reductive dehalogenases are preceded by twin arginine signal-sequences, which are found in periplasmic proteins and often involved in membrane-associated electron transfer. Also confirmed is the presence of iron–sulfur clusters, which are characteristic of bacterial ferredoxin.

To date, *Dehalococcoides ethenogenes* strain 195 is the only strain known that is able to completely dechlorinate PCE to ethene [8]. Bacterial growth by halorespiration of chloroethenes, chlorobenzenes, 3-chlorobenzoate and 2-chlorophenol has been well documented [1,9]. However, bacterial growth by reductive dechlorination of TCA has not been reported, although biotransformation of TCA has been observed under aerobic and anaerobic conditions in co-metabolic processes. Sun *et al.* recently isolated a novel halorespiring anaerobic bacterium, strain TCA1, that requires hydrogen as an electron donor and TCA as an electron acceptor for growth [8]. TCA is a ubiquitous environmental pollutant, as are PCE and TCE, because of their widespread use as industrial solvents. Strain TCA1 is capable of conserving energy for growth through the reductive dechlorination of TCA, converting TCA sequentially to 1,1-dichloroethane and chloroethane. The closest relative of strain TCA1 is *Dehalobacter restrictus* strain TEA [10], which has a sequence similarity of 99%. *D. restrictus* is a strict anaerobe capable of coupling PCE and TCE dechlorination to hydrogen oxidation for growth in a respiratory process. Although *in situ* bioremediation for the chloroethenes is well known, TCA bioremediation remains to be established. The potential of strain TCA1 to detoxify TCA in the natural environment through bioaugmentation was ascertained for anoxic aquifer sediments [11]. TCA was completely converted to chloroethane within two months in aquifer sediment samples inoculated with strain TCA1, whereas no dechlorination was observed in samples without the inoculum. These results suggest that bioaugmentation with strain TCA1 could ensure the degradation of TCA because chloroethane can be degraded easily under aerobic conditions. Bunge *et al.* showed the presence of a strictly anaerobic

Dehalococcoides species in four dioxin-dechlorinating environment cultures from freshwater sediment highly contaminated with dibenzo-*p*-dioxins and dibenzofurans [12]. They also demonstrated that the previously described chlorobenzene-dehalorespiring bacterium *Dehalococcoides* sp. strain CBDB [13] is able to reductively dechlorinate selected dioxin congeners. For instance, 1,2,3,7,8-penta-chlorodibenzo-*p*-dioxin was reductively dechlorinated to 2,7- or 2,8-dichlorodibenzo-*p*-dioxin, indicating that environmentally significant dioxins are attacked by this anaerobic bacterium.

Attempts to enhance PCB biodegradation

PCBs are complex mixtures containing between one and ten chlorines on a biphenyl molecule, and theoretically 209 different compounds are produced by direct chlorination. Thus, the biodegradability of PCBs is highly influenced by chlorine substitution and, in addition, the biodegradation capability of bacteria is highly strain dependent. PCBs are transformed by a co-metabolic process using biphenyl-catabolizing enzymes to form chlorobenzoates [2]. Four enzymes are involved in this reaction, including: biphenyl dioxygenase (Bph Dox), which introduces molecular oxygen to one of the biphenyl rings, usually at the 2 and 3 positions; a dehydrogenase; dihydroxybiphenyl dioxygenase (DHBD), which cleaves the biphenyl ring; and a hydrolase.

It has been repeatedly reported that *ortho*-chlorinated PCBs such as 2,2-, 2,6-, 2,3,6- and 2,4,6-chlorobiphenyls are recalcitrant to microbial attack and hydroxylated intermediates often accumulate. The reason why such *ortho*-chlorinated PCBs are so resistant to microbial degradation has remained unknown. Dai *et al.* recently identified and analyzed a bottleneck in PCB biodegradation [14]. They investigated the specificity of DHBD, as well as the involvement of inhibition and inactivation as limiting factors *in vitro* and *in vivo*. The results of these studies revealed that *ortho*-chlorinated metabolites strongly inhibit DHBD, promote its suicide inactivation and interfere with the degradation of other PCB compounds. The apparent k_{cat} for 2,6-dichlorodihydroxybiphenyl was reduced by a factor of ~7000 relative to nonchlorinated 2,3-dihydroxybiphenyl. It bound with sufficient affinity to completely inhibit dihydroxybiphenyl cleavage at nanomolar concentrations. Furthermore, they solved the crystal structures of two complexes of DHBD with *ortho*-chlorinated metabolites at 1.7 Å resolution, to explain these inhibitory effects.

Attempts to expand and enhance the range of PCB degradation have recently been conducted successfully in our laboratory and others. The large subunit (BphA1) of Bph Dox (encoded by *bphA1*) was targeted in these studies because this component is responsible for substrate specificity. Kumamaru *et al.* first succeeded in generating the chimeric Bph Dox with enhanced capacities for PCB degradation using DNA shuffling between the two *bphA1* genes encoding proteins of which the structures are similar but differ functionally [15]. Similar DNA shuffling approaches were used to evolve a Bph Dox that exhibits superior degradation capabilities for highly chlorinated PCBs and persistent 2,6-dichlorobiphenyl [16,17].

Based on crystallographic analyses of naphthalene dioxygenase, Suenaga *et al.* developed a 3D model of BphA1 [18]. They constructed a variety of site-directed BphA1 mutants with changes of amino acids that coordinate the catalytic non-heme iron center. The resulting modified Bph Dox exhibited altered regiospecificities for various PCBs compared with wild-type Bph Dox.

Hybrid enzyme for effective TCE degradation

As oxygenases generally possess relaxed substrate specificity, some oxygenases, such as methane monooxygenase, toluene dioxygenase, toluene monooxygenase and phenol hydroxylase, are known to degrade TCE by co-metabolism. A gene cluster of toluene dioxygenase (Tol Dox) encoded by *todC1C2BA* of *Pseudomonas putida* F1 and *bphA1A2A3A4* coding for Bph Dox of *Pseudomonas pseudoalcaligenes* KF707 are similar in gene organization and nucleotide sequences. The identities of amino acid sequences between the corresponding subunits of Bph Dox and Tol Dox are between 53% and 65%. Bph-Tod hybrid dioxygenases were constructed and expressed in *Escherichia coli*, where the genes encoding large and/or small subunits from two dioxygenases were replaced with each other [19]. *E. coli* cells expressing the hybrid dioxygenase composed of TodC1-BphA2-BphA3-BphA4 degraded TCE efficiently compared with those with the original Bph Dox and Tol Dox. Maeda *et al.* purified the individual subunit components of the Bph-Tod hybrid dioxygenase and reconstituted these for TCE degradation, demonstrating that the large and small subunit are associated as $\alpha\beta\beta$ and that its increased affinity for TCE might lead to a higher TCE degradation capability [20].

Furthermore, hybrid *Pseudomonas* strains in which the *bphA1* gene is replaced by *todC1* within the chromosomal *bph* gene clusters acquired the novel ability of growing on single-ring aromatic hydrocarbons such as benzene and toluene, and retained this growth capability on biphenyl [21]. More interestingly, these hybrid strains degraded TCE very efficiently. The same strains degraded *cis*-1,2-dichloroethylene (*cis*-1,2-DCE) faster than TCE.

We demonstrated effective and total degradation of PCE using the anaerobic *Desulfitobacterium* strain Y51 producing PCE dehalogenase [4,7] and the hybrid *Pseudomonas* strain producing Bph-Tod dioxygenase [21]. Thus, PCE (at an initial concentration of up to 100 $\mu\text{g ml}^{-1}$) was converted to *cis*-1,2-DCE by strain Y51 anaerobically, and the *cis*-1,2-DCE produced was degraded oxidatively by the hybrid *Pseudomonas* strain.

The discovery of an anaerobic halorespiring strain that couples reductive dehalogenation of chlorinated organic compounds to growth...suggests a strategy for effective bioremediation.

Conclusion

The discovery of an anaerobic halorespiring strain that couples reductive dehalogenation of chlorinated organic compounds to growth not only leads to a better understanding of the physiology, biochemistry and molecular genetics of halorespiring bacteria, but also suggests a strategy for effective bioremediation. Directed evolution and the rational design of genes are exciting tools for the enhancement of enzymes not only for the degradation of recalcitrant compounds, but also for the production of useful chemicals [22]. Modified degradative genes could be introduced into the original strains and/or major indigenous strains isolated from contaminated sites, and it is hoped that these 'super bugs' could have applications in bioremediation in the near future, confirming their usefulness and safety.

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Transgenesis and yield: what are our targets?

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Plant metabolic engineering has been used to generate a wide range of transgenic lines in which specific metabolic steps have been targeted. Attempts to increase yield of some agronomically important crops using this approach have highlighted the inherent complexities of modulating plant metabolism. In light of the recent findings by Regierer *et al.* this article addresses the major challenges faced with respect to enhancing yield through transgenesis.

The first use of transgenesis in plants in the 1980s heralded the arrival of a powerful and exciting tool for the study of metabolic regulation and for crop improvement. Of particular interest from a commercial viewpoint was the potential for increasing yield thus making the alteration of carbon partitioning between sucrose, starch and amino acids an important target for manipulation.

During the past 15 years a high proportion of the transgenic lines that have been generated has highlighted the problems associated with effective manipulation of plant metabolism. Plant metabolism displays plasticity and often compensates for perturbations resulting from metabolic manipulations. The complex interactions of metabolic pathways, and our poor understanding of regulatory mechanisms, mean that targets for manipulation are not always obvious. Attempts to modify metabolism in specific ways have highlighted several key considerations. First, the alteration of the level of specific metabolites may have knock-on effects throughout metabolism because each metabolite may act as a substrate for several branches of metabolism as well as an effector molecule to enzymes in seemingly unrelated pathways [1,2]. This emphasizes the importance of an understanding of the regulatory properties of individual enzymes and the control of pathways prior to their manipulation. Second, it is possible that the plant can compensate for metabolic changes by using an alternative enzyme to catalyse the same reaction [3], or that a particular enzyme will be present in excess so that large reductions in its levels will have little effect on metabolism [4]. Finally, plants can silence the expression of transgenes via methylation, thus preventing or reducing their effect on metabolic processes [5]. Attempts to improve yield have relied mainly on

assumptions as to which factors might be important for metabolite partitioning based on our 'classical' views of metabolic pathways. Some improvements in yield have been achieved but the success rate has been low compared with our initial expectations.

Identifying targets

Several approaches have been taken to increase yield using transgenic technology. These include manipulating metabolism in source tissues with the aim of increasing carbon supply to heterotrophic sink tissues [6]; increasing transport capacities between source and sink tissues to try to increase photoassimilate supply to the sink tissues [7] and manipulating metabolism in the sink tissue to increase the utilization of photoassimilates thereby increasing amounts of specific compounds [1]. These approaches used either ectopic overexpression of heterologous genes or antisense repression of genes. Molecular biology, trait mapping and targeted gene reduction using transgenesis coupled with metabolic control analysis have accelerated our understanding of key metabolic pathways over the past 10 years. This has resulted in a revision of our ideas about the regulation of metabolism, so we can now be more rational about which enzymes are likely to have an impact on partitioning, and therefore on yield. The potato tuber in particular has been a focus of biochemical characterisation and manipulation owing to its importance as a starch-storing crop, the genetic resources available for its transformation and the relative ease with which transgenic potato plants can be produced.

Regierer *et al.* recently published an example of the use of improved biochemical knowledge to achieve increases in yield [8]. They report a 60% increase of starch in potato tubers, a 39% increase in tuber yield and an increase in the concentration of some amino acids above wild-type levels. This was achieved by transgenically down-regulating the activity of the plastidial isoform of adenylate kinase, with the aim of altering adenylate pools, which have been shown to be important for starch synthesis [9]. This represents the largest increase in starch resulting from transgenesis observed to date, and introduces a novel approach for increasing yield which involves modulating the concentration of cofactors, metabolites that are involved in many more reactions than the one being

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