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13. ABSTRACT (Maximum 200 words)

Sphingobium chlorophenolicum is a Gram-negative soil bacterium that can mineralize pentachlorophenol (PCP), although degradation is slow and the bacterium cannot tolerate high levels of PCP. The rate of degradation is limited by the first enzyme in the pathway, PCP hydroxylase, which is a very poor catalyst. We have used DNA shuffling to produce mutant enzymes with modestly improved catalytic activities. We discovered that the reason for our limited success was that our selection was based upon publications claiming that the product formed by PCP hydroxylase is tetrachlorohydroquinone (TCHQ). Since TCHQ is less toxic than PCP, cells expressing better enzymes should be able to grow in the presence of higher levels of PCP. However, we found that the product formed from PCP is actually tetrachlorobenzoquinone (TCBQ), which is more toxic than PCP. We also discovered a previously unrecognized enzyme that converts TCBQ to TCHQ. Our new knowledge about the pathway will allow us to design a more effective selection for improved PCP hydroxylase enzymes. Finally, we have used genome shuffling to generate strains of S. *chlorophenolicum* that have greatly increased tolerance to the toxicity of PCP and have significantly improved rates of degradation.

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The pathway for degradation of pentachlorophenol (PCP) provides an important opportunity to study the process of molecular evolution in bacteria that are exposed to a novel and toxic pesticide. My research program is directed at understanding the origin of the enzymes that have been recruited to serve new functions in this pathway, how they catalyze new reactions, how well they work, and how they can be improved. The goals of this project were to analyze the kinetic abilities of PCP hydroxylase, to identify the factors that limit the ability of the enzyme to catalyze the reaction, and to use *in vitro* evolution methods to evolve a new enzyme. The project evolved during the granting period, primarily as a result of the discovery that the product formed by PCP hydroxylase was previously misidentified, and that the pathway contained a previously unrecognized enzyme. In addition, we took advantage of a novel technology to create a bacterial strain with significantly improved degradative abilities. This report will describe progress made in three different areas.

Efforts to Create a Better PCP Hydroxylase using DNA Shuffling

In vitro evolution procedures[1,2,3] introduced in the last decade offer a powerful way to evolve enzymes with altered specificity, catalytic parameters, and physical properties. In these methods, a large library of mutant genes is created using either DNA shuffling or error-prone PCR to introduce genetic diversity. A selection or screen is then used to identify variants with the desired property. We initiated attempts to evolve a better PCP hydroxylase three years ago. Genetic diversity was introduced using DNA shuffling. Our plan was to select for better variants of PCP hydroxylase by transforming the library into *E. coli* and selecting cells that were more resistant to the toxicity of PCP. At the time, the product formed from PCP by PCP hydroxylase was reported to be tetrachlorohydroquinone (TCHQ),[4,5] which is substantially less toxic than is PCP. *E. coli* JM105 cannot grow in the presence of PCP at concentrations higher than 100 μg/ml. When a plasmid encoding the wild-type PCP hydroxylase is introduced into *E. coli* JM105 and expression of the hydroxylase is induced with IPTG, the bacteria can grow at concentrations of 600 μg/ml PCP. In the presence of an improved PCP hydroxylase, we expected that the cells would be able to grow at even higher levels of PCP.

After repeated rounds of DNA shuffling, we were only able to evolve enzymes with a very modest improvement in k_{cat} (see Table 1). Since the k_{cat} of PCP hydroxylase is about three orders of magnitude lower than those of most phenol hydroxylases, we expected to be able to achieve a much higher level of activity. We now understand that the strategy was flawed: the product formed from PCP by PCP hydroxylase is actually tetrachlorobenzoquinone (TCBQ), which is more toxic than PCP. Thus, the most effective enzymes would produce a large amount of TCBQ, which would overwhelm and kill the cells.

Table 1. Summary of activity and amino acid substitutions found in improved PCP hydroxylase enzymes generated by DNA shuffling.

Enzyme	k_{cat} (s^{-1})	substitution
wt	0.02	
M9	0.08	S177T
M22	0.13	L295W, L525F
MG	0.05	D173V, V175G
MJ	0.06	R176A, R155M, G171R
M31	0.08	S177N, A72V, A73V, S101F, F109V, D212N
M52	0.07	S165F, D158G
MC	0.03	G355D

Discovery of a Previously Unrecognized Step in the Pathway Catalyzed by PcpD

The gene encoding PCP hydroxylase (*pcpB*) is immediately upstream of two additional genes (see Figure 1).[6] *pcpR* encodes a regulatory protein that responds to PCP. *pcpD,* which is immediately downstream of *pcpB*, resembles genes for the reductase components of two-component dioxygenases, some of which hydroxylate aromatic compounds. Based upon this resemblance, it was proposed that PcpD is a reductase that facilitates the hydroxylation of PCP by PCP hydroxylase,[6] and the annotation of PcpD in GenBank states that it is pentachlorophenol 4-monooxygenase reductase.

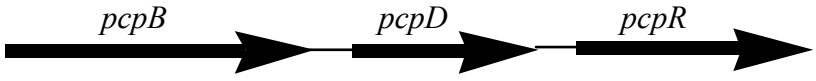

Figure 1. Organization of *pcpB, pcpD* and *pcpR.*

We suspected that this assignment was incorrect because PCP hydroxylase is a flavin monooxygenase, and such enzymes do not generally require reductases. Consequently, we undertook studies to determine whether PcpD is required for degradation of PCP. We found that transcription of *pcpD* is induced by PCP, as previously reported for *pcpA* and *pcpB*. A mutant strain in which PcpD was knocked out was able to remove PCP from the medium at low concentrations, but not at high concentrations. In contrast, the knockout strain could remove tetrachlorophenol (TCP) from the medium as well as the wild type strain even at high concentrations. These results suggested that PcpD may catalyze a step that is critical for degradation of PCP but not TCP, and therefore must involve the chlorine at the 4-position of PCP. Careful consideration of the expected mechanism for PCP hydroxylase suggested a possible explanation (see Figure 2). Hydroxylation of a substrate that bears a chlorine at the site of hydroxylation should produce a benzoquinone product due to gem elimination of HCl from the initial hydroxylation product. However, hydroxylation of a substrate with a hydrogen at the position of hydroxylation leads to a hydroquinone. Thus, an enzyme that reduces TCBQ should be required for degradation of PCP, but not TCP. Consequently, we proposed and verified that PcpD is a TCBQ reductase. Further studies showed that the inability of the knockout strain to remove PCP from the medium at high concentrations can be attributed to inactivation of PCP hydroxylase by TCBQ that accumulates in the absence of PcpD. This work was published in the *Journal of Bacteriology* in 2003.[7]

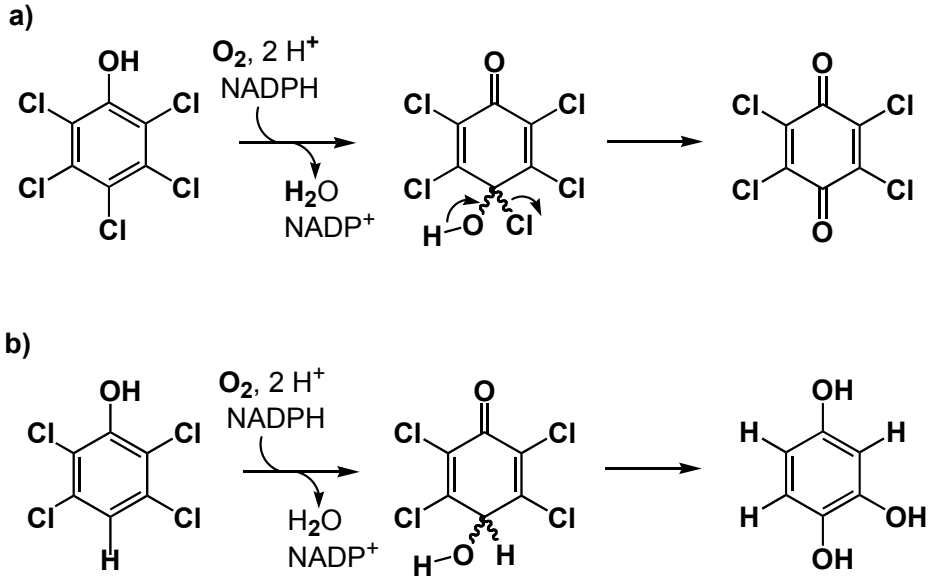

Figure 2. Hydroxylation of a phenol produces a benzoquinone when hydroxylation occurs at a position bearing a chlorine (a), but a hydroquinone when hydroxylation occurs at a position bearing a hydrogen (b).

The discovery that PcpD is TCBQ reductase requires an important revision in the pathway, which had previously been reported to proceed from PCP directly to TCHQ.[4, 5] Furthermore, this discovery has provided important insights into two questions. First, it explains why our initial attempts to improve PCP hydroxylase were only modestly successful. Second, it explains why *S. chlorophenolicum* has not been able to evolve a more effective PCP hydroxylase that would allow greater flux of carbon through the metabolic pathway. If a mutation that led to a highly active PCP hydroxylase were to occur in nature, the increased production of TCBQ might overwhelm the abilities of TCBQ reductase, and those cells would not survive. If a mutation that led to an improved TCBQ reductase occurred, those cells would not necessarily be more fit since the flux through the pathway would still be limited by the poor PCP hydroxylase. In order to improve the flux through the pathway, a cell would have to simultaneously improve both enzymes. This is extremely unlikely, especially if an improvement in either enzyme requires more than mutation, which is likely to be the case. Thus, we expect that improvements in these enzymes will be very difficult to achieve in nature.

Improvement of PCP Degradation Using Genome Shuffling

PCP can be mineralized by *S. chlorophenolicum*, but degradation of PCP is slow, and *S. chlorophenolicum* cannot tolerate high levels of PCP. We have used genome shuffling to improve the degradation of PCP by *S. chlorophenolicum*. Genome shuffling involves generation of mutant strains that have an improved phenotype, followed by multiple rounds of protoplast fusion to allow recombination between genomes. This approach has recently been used to improve production of the polyketide antibiotic tylosin in *Streptomyces fradiae*[8] and to improve acid tolerance in *Lactobacillus*.[9] Genome shuffling is useful for engineering of multi-trait phenotypes that would be difficult to engineer directly because it may be impossible to anticipate all of the mutations needed to improve a complex trait while still maintaining robust growth. The improvement of PCP degradation is an excellent problem to be

addressed by this methodology. Because we expect that it may be difficult to improve flux through the pathway, adaptations that reduce the toxic effects of PCP and its metabolites are likely to be particularly important, and these are likely to require multiple mutations. We have obtained several strains that degrade PCP faster and tolerate higher levels of PCP than the wild type strain. Several strains obtained after the third round of shuffling can grow on 1/4 strength tryptic soy broth plates containing 6-8 mM PCP, while the original strain cannot grow in the presence of PCP at concentrations higher than 0.6 mM. Some of the mutants are able to completely degrade 3 mM PCP in liquid 1/4 strength tryptic soy broth, whereas no degradation can be achieved by the wild type strain. Analysis of several improved strains suggests that the improved phenotypes are due to various combinations of mutations leading to enhanced growth rate, constitutive expression of the PCP degradation genes, and enhanced resistance to the toxicity of PCP and its metabolites. These results suggest that improved degradation can be achieved in a number of different ways, an issue that has not heretofore been explored in reports of genome shuffling experiments. This work has been accepted for publication in *Applied and Environmental Microbiology*.

Publications resulting from this grant

Dai, M.; Bull Rogers, J.; Warner, J. R.; Copley, S. D. "A previously unrecognized step in pentachlorophenol degradation in *Sphingobium chlorophenolicum* is catalyzed by tetrachlorobenzoquinone reductase (PcpD)." *J. Bacteriol.* **2003**, *185*, 302-310.

Dai, M.; Copley, S. D. "Genome Shuffling Improves Degradation of the Anthropogenic Pesticide Pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723.", *Appl. Env. Microbiol.* In press.

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