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Evolution of Enzymes Required for Biodegradation of Pentachlorophenol in Sphingobium Chlorophenolicum

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## Report Title

Evolution of Enzymes Required for Biodegradation of Pentachlorophenol in Sphingobium chlorophenolicum - Final Report


## ABSTRACT

Pentachlorophenol (PCP) is a highly toxic pesticide first introduced into the environment in 1936. PCP can be mineralized by Sphingobium chlorophenolicum. However, biodegradation is slow and the bacterium cannot tolerate high levels of PCP. Flux through the pathway is limited by the first enzyme, PCP hydroxylase, which converts PCP to tetrachlorobenzoquinone (TCBQ) very slowly. Furthermore, it catalyzes a futile cycling reaction in which C4a-hydroperoxyflavin at the active site decays to produce H2O2 without hydroxylating the substrate, thus wasting NADPH and producing a toxic by-product. The enzyme also hydroxylates tetrachlorohydroquinone, a downstream metabolite. This reaction generates TCBQ, effectively reversing the normal direction of the pathway. Finally, TCBQ forms an adduct with the flavin and inactivates the enzyme. The enzyme has clearly not yet evolved to be an effective catalyst for hydroxylation of PCP. Improvements in the ability of Sphingobium chlorophenolicum to degrade PCP can be generated using genome shuffling. We created several strains that degraded PCP faster and tolerated higher concentrations of PCP than the wild type strain. Notably, none of the strains contained an improved version of PCP hydroxylase, suggesting that mutations that affect other processes can have an important effect on the efficiency of the degradation pathway.


## List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

### (a) Papers published in peer-reviewed journals (N/A for none)

Dai, M. H., Zeisman, S., Ratcliffe, T., Gill, R. T., and Copley, S. D. “Visualization of Protoplast Fusion and Quantitation of Recombination in Fused Protoplasts of Auxotrophic Strains of Escherichia coli”, Metabolic Engineering 7, 45-52, 2005

Dai, M.-H. and Copley, S. D. “Genome Shuffling Improves Degradation of the Anthropogenic Pesticide Pentachlorophenol by Sphingobium chlorophenolicum ATCC 39723”, Appl. Env. Microbiol. 70, 2391-2397, 2004.

**Number of Papers published in peer-reviewed journals:** 2.00

### (b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

**Number of Papers published in non peer-reviewed journals:** 0.00

### (c) Presentations

“Gene Recruitment and the Evolution of PCP Degradation”, invited talk presented at the 106th General Meeting of the American Society for Microbiology, Orlando, Florida, May 21-25, 2006.

“Pentachlorophenol hydroxylase: Havoc at the active site”, poster presentation at the Gordon Research Conference on Enzymes, Coenzymes, and Metabolic Pathways, Biddeford, Maine, July, 2006.

**Number of Presentations:** 2.00

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**Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):** 0

### (d) Manuscripts

Pietari, J. M. H., Behlen, L. S. and Copley, S. D. "Pentachlorophenol hydroxylase: havoc at the active site”, submitted to Biochemistry.

**Number of Manuscripts:** 1.00

**Number of Inventions:**

## Graduate Students

| NAME | PERCENT_SUPPORTED |
|---|---|
| Ashley Trahan | 0.50 |
| Joe Warner | 0.08 |
| **FTE Equivalent:** | **0.58** |
| **Total Number:** | **2** |

## Names of Post Doctorates

| NAME | PERCENT_SUPPORTED |
|---|---|
| Jaana Pietari | 1.00 |
| MingHua Dai | 1.00 |
| **FTE Equivalent:** | **2.00** |
| **Total Number:** | **2** |

## Names of Faculty Supported

| NAME | PERCENT_SUPPORTED | National Academy Member |
|---|---|---|
| Shelley D. Copley | 0.08 | No |
| **FTE Equivalent:** | **0.08** | |
| **Total Number:** | **1** | |

## Names of Under Graduate students supported

| NAME | PERCENT_SUPPORTED |
|---|---|
| Sarah Urfer | 0.25 |
| **FTE Equivalent:** | **0.25** |
| **Total Number:** | **1** |

## Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

| | |
|---|---|
| The number of undergraduates funded by this agreement who graduated during this period: ..... | |
| The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields: ..... | 1.00 |
| The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields: ..... | 1.00 |
| Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): ..... | 0.00 |
| Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering: ..... | 0.00 |
| The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense ..... | 0.00 |
| The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: ..... | 0.00 |

## Names of Personnel receiving masters degrees

| NAME |
| --- |
| Ashley Trahan |
| **Total Number:** 1 |

## Names of personnel receiving PHDs

| NAME |
| --- |
| **Total Number:** |

## Names of other research staff

| NAME | PERCENT_SUPPORTED | |
| --- | --- | --- |
| Linda Behlen | 0.08 | No |
| **FTE Equivalent:** | **0.08** | |
| **Total Number:** | **1** | |

## Sub Contractors (DD882)

## Inventions (DD882)

## Scientific Accomplishments

### *Foreword*

We are studying the biodegradation of pentachlorophenol (PCP), an anthropogenic pesticide first introduced into the environment in 1936. Biodegradation of PCP is particularly challenging because it uncouples oxidative phosphorylation and alters membrane fluidity. Despite its toxicity and recent introduction into the environment, PCP can be completely degraded by *Sphingobium chlorophenolicum*, several strains of which have been isolated from PCP-contaminated soil. Although the ability of *S. chlorophenolicum* to mineralize PCP is remarkable, the inefficiency of the metabolic pathway has limited the potential for its use in bioremediation. The initial part of the degradation pathway is shown in Figure 1. Our major accomplishments include 1) characterizing the reasons for the poor catalytic performance of the first enzyme in the pathway, PCP hydroxylase; and 2) improving the ability of *S. chlorophenolicum* to degrade PCP using genome shuffling.

### *List of Illustrations*



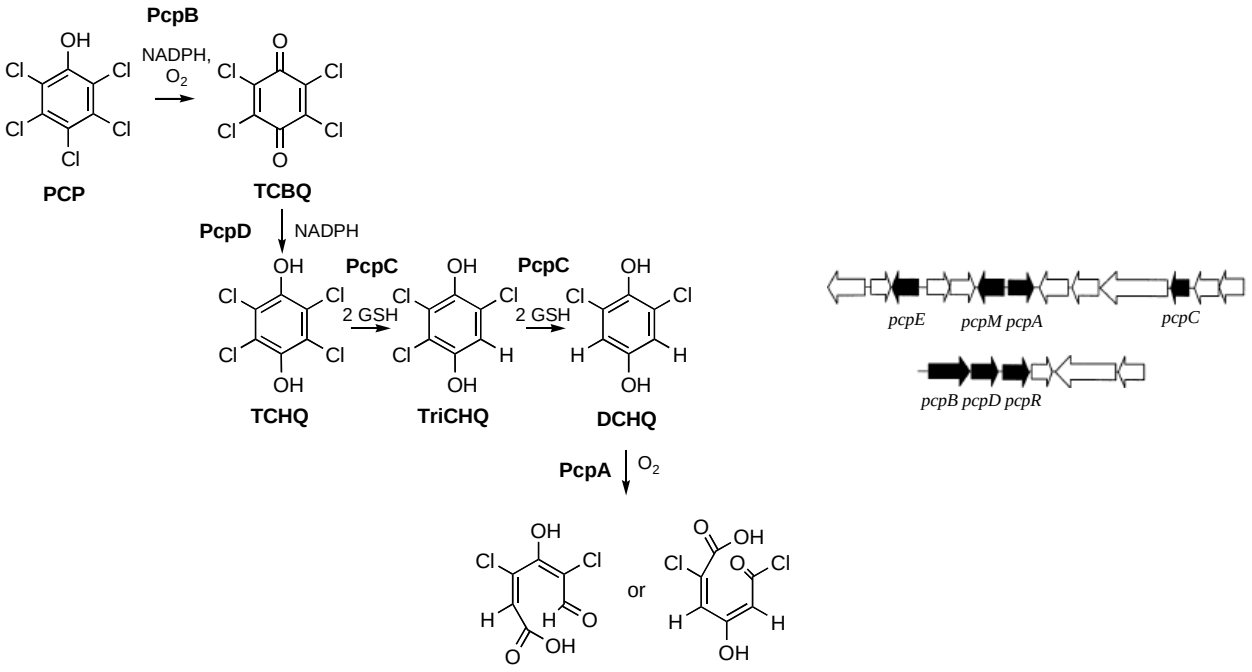


Figure 1. (left) Pathway for degradation of PCP in *S. chlorophenolicum* ATCC 39723. PcpB, PCP hydroxylase; PcpD, TCBQ reductase; PcpC, TCHQ dehalogenase; PcpA, DCHQ dioxygenase (PcpA); GSH, glutathione. (right) Organization of genes involved in PCP degradation.[1]

*Investigation of the Catalytic Inefficiency of PCP Hydroxylase*

Previous work suggests that the rate-limiting step for degradation of PCP is the first step in the pathway, which is catalyzed by PCP hydroxylase.[2] This enzyme is an extremely poor catalyst. The $k_{cat}$ for turnover of PCP is 0.05 $s^{-1}$, and the $K_M$ is approximately 0.5 mM. Thus, $k_{cat}$ / $K_M$ is about $10^5$ $M^{-1}s^{-1}$. (For comparison, $k_{cat}$ values for typical flavoprotein hydroxylases range between 4 and 57 $s^{-1}$,[3,4,5,6,7] and $k_{cat}/K_M$ values are typically $10^5$ - $10^6$ $M^{-1}s^{-1}$.[4,7]) Since the enzyme is apparently saturated with PCP *in vivo*, the low $k_{cat}$ limits the flux through the pathway.

Dr. Jaana Pietari explored the reasons for the poor catalytic performance of PCP hydroxylase. She found that the enzyme is unable to efficiently couple formation of the $C4_a$-hydroperoxyflavin intermediate with hydroxylation of the substrate. About 75% of the $C4_a$-hydroperoxyflavin eliminates $H_2O_2$ rather than hydroxylating the substrate, resulting in production of a toxic product and wasting valuable NADPH (see Figure 2). This type of "futile cycling" has been observed with other phenol hydroxylases when poor substrates or analogues that cannot be hydroxylated are used. Futile cycling also occurs at the active site of PCP hydroxylase when tetrachlorohydroquinone (TCHQ), a downstream metabolite, is incubated with the enzyme. TCHQ also appears to be hydroxylated to some extent at the active site. Hydroxylation of TCHQ produces an unstable product that generates TCBQ, in effect a step backwards in the metabolic pathway.

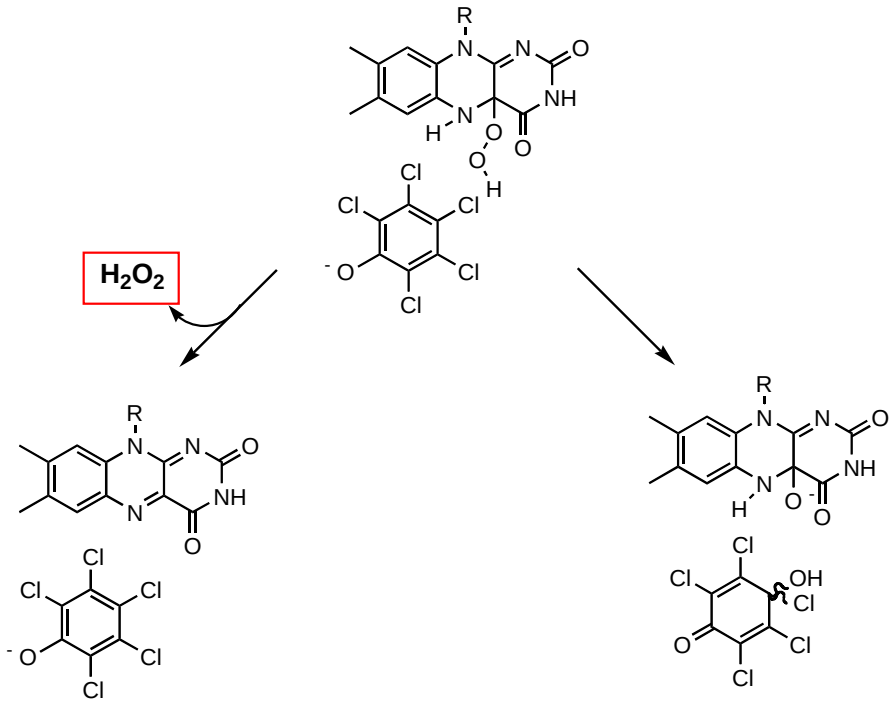


Figure 2. Two alternative fates of the C4a-hydroperoxyflavin formed by reaction of $O_2$ with the reduced flavin at the active site of PCP hydroxylase. Right) a hydroxyl group can be transferred to the substrate; left) $H_2O_2$ can be eliminated when hydroxylation of the substrate is very slow.

TCBQ, the product formed from PCP, inactivates the enzyme.[8] Incubation of the enzyme with only a 5-fold excess of TCBQ for 30 minutes causes loss of 90% of the activity. We have discovered that the reason for the loss of activity is that TCBQ forms a covalent adduct with the flavin. Furthermore, the flavin can no longer be removed from the enzyme by heat treatment, suggesting that a covalent adduct has formed between the flavin and the protein, probably mediated by the TCBQ, has formed. This adduct does not form when NADPH is present. Thus, *in vivo*, the enzyme would not be inactivated until futile cycling had depleted the cytoplasmic supply of NADPH.

The results we have obtained are summarized in Figure 2. A manuscript describing these results has been submitted to *Biochemistry*. We conclude that PCP hydroxylase has not yet evolved an active site capable of controlling the reactivity of the $C4_a$-hydroperoxyflavin to prevent futile cycling and of hydroxylating PCP at a high rate. This new understanding of the reasons for the poor catalytic performance of PCP hydroxylase will inform our future efforts to engineer an improved version of the enzyme.

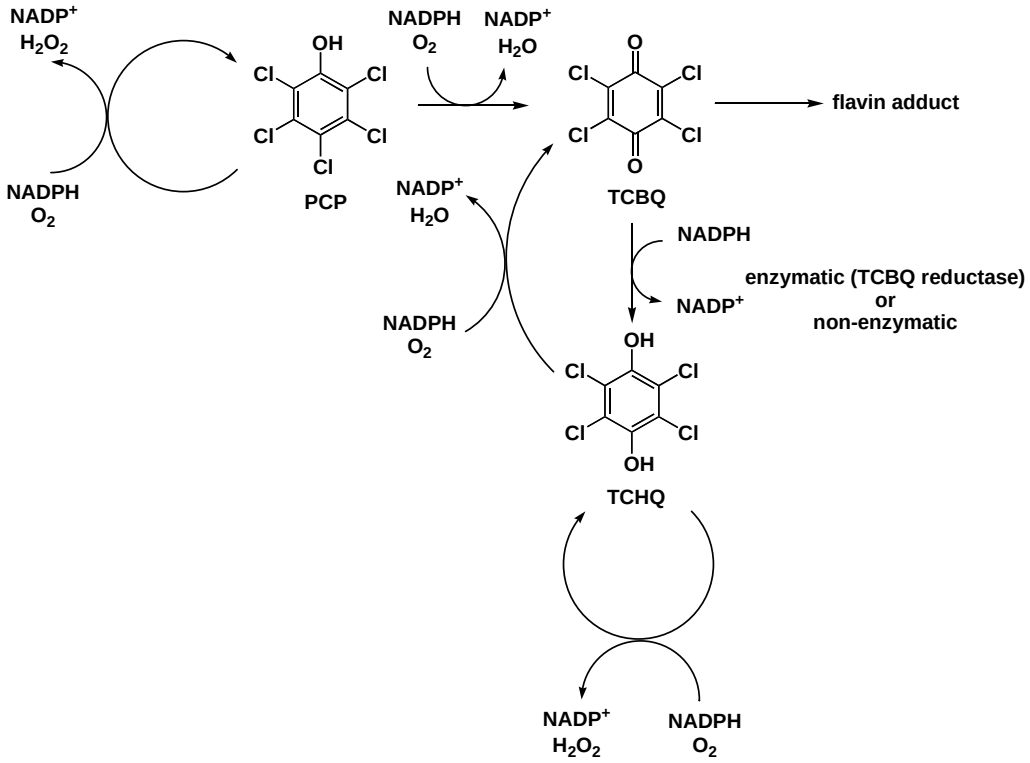


Figure 3. Summary of the processes catalyzed by PCP hydroxylase.

### *Investigation of the Origin of TCBQ Reductase*

TCBQ reductase catalyzes the second step in the pathway, the reduction of TCBQ to TCHQ. We discovered this enzyme during the previous grant period.[8] The evolutionary origin of this enzyme is interesting because TCBQ reductase is not related to known quinone reductases. Quinone reductases in the respiratory chains of all types of cells transfer electrons to mobile quinone electron carriers such as ubiquinone. There are also two families of soluble

quinone reductases. One family catalyzes direct reduction of quinones to hydroquinones by NADH.[9] The other family transfers electrons from NADH to a flavin and then to a quinone.[10,11] TCBQ reductase is not related to any of these, but rather to the reductase components of two-component dioxygenases (specifically the Class I reductases).[12] These proteins contain a flavin and a plant-type [2Fe-2S] ferredoxin and transfer electrons from NADPH to an iron sulfur cluster on the oxygenase, which catalyzes attack of $O_2$ on an aromatic substrate. Our goal is to understand how and why a protein related to this family of reductases was recruited to serve as a TCBQ reductase.

Progress on this project was slow because it is difficult to express TCBQ reductase in soluble form in *E. coli*. We transferred the *pcpD* gene into several expression vectors using the Gateway technology and succeeded in expressing soluble TCBQ reductase in *E. coli* using a vector (pBAD-Dest49) that adds an N-terminal His-patch thioredoxin tag and a C-terminal His tag to the protein. The protein can be purified using a nickel affinity resin, and thioredoxin tag cleaved with enterokinase. This procedure will allow us to carry out a detailed kinetic characterization of TCBQ reductase.

### *Genome Shuffling Improves Degradation of PCP by Sphingobium chlorophenolicum*

Dr. MingHua Dai explored the use of genome shuffling to improve 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.[13,14] 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.

*S. chlorophenolicum* strain ATCC 39723 cannot grow on plates containing PCP at concentrations higher than 0.6 mM. In order to obtain mutants with an improved ability to grow in the presence of PCP, *S. chlorophenolicum* was treated with nitrosoguanidine and then plated on 1/4 strength tryptic soy broth (TSB) containing 1.6 mM PCP. The thirty-four colonies obtained were used as the starting population for genome shuffling. Three successive rounds of protoplast fusion were carried out, and after each round, the concentration of PCP in the plates used for selection was increased. At the conclusion of the shuffling, the ability of strains isolated during all three rounds to grow in the presence of PCP in liquid medium was assessed, and the best two or three strains from each round were selected for further characterization. This process resulted in substantial improvements in both the rate of PCP degradation (see Figure 3) and the concentration of PCP that can be tolerated (not shown). Analysis of several improved strains indicates that 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 contribute to the improved phenotypes. These results suggest that enhanced performance can be achieved in a number of different ways, an issue that had not been explored in previous reports of genome shuffling experiments. This work was published in *Applied and Environmental Microbiology.* We subsequently adapted the methods we had used for use in *E. coli* and published an additional paper in *Metabolic Engineering.*

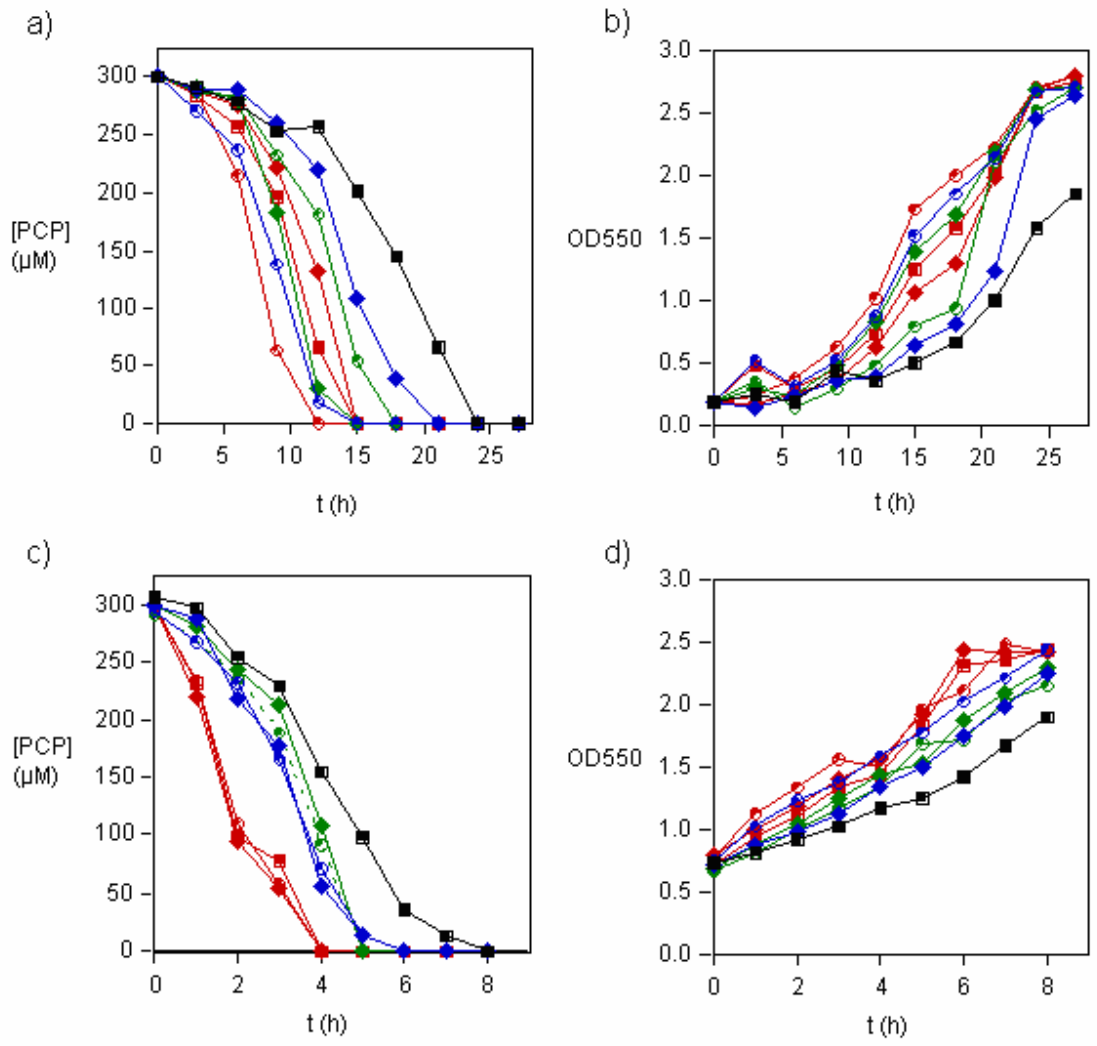


Figure 3. Degradation of PCP by and cell growth of wild type and mutant strains of *S. chlorophenolicum* obtained by genome shuffling. Strains 108 and 130 were obtained from the first round of shuffling, strains 206 and 215 from the second, and strains 307, 316, and 320 from the third. Degradation of 0.3 mM PCP by (a) and cell growth of (b) cells not previously exposed to PCP; degradation of 0.3 mM PCP by (c) and cell growth of (d) cells previously exposed to 50 μM PCP; (■) wild type; (◆) strain 108; (●) strain 130; (◆) strain 206; (●) strain 215; (◆) strain 307; (●) strain 316; (■) strain 320. For the experiments in panels c and d, cells were grown in the presence of 50 μM PCP, and additional PCP was added when the $OD_{550}$ reached 0.6.

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