Making Better Poison Eaters: Metabolic Engineering for Bioremediation

by

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Blue Team: Speed and Toxicity

Bioremediation is defined as the use of microorganisms to remove pollutants, such as pentachlorophenol (PCP), from the environment. The *National Geographic* article "Mediocre Poison Eaters and the Imperfection of Evolution" (Zimmer, 2013) describes the inefficiency of PCP degradation (Figure 1B), and it is clear that this metabolic pathway has not been optimized in nature to degrade PCP. Our goal as scientists is to create a strain of *Sphingobium chlorophenolicum* that can more efficiently break down PCP for use in bioremediation applications.

There are three interconnected considerations when engineering your improved strain:

1. Improving flux through the pathway to decrease exposure to toxic intermediates.
2. Balancing metabolites so that PCP degradation can happen alongside normal metabolism.
3. Decreasing the potential for PcpA inhibition through enzyme inhibition.

Blue team goal: Improve the rate of PCP degradation through the pathway while decreasing exposure to toxic compounds.

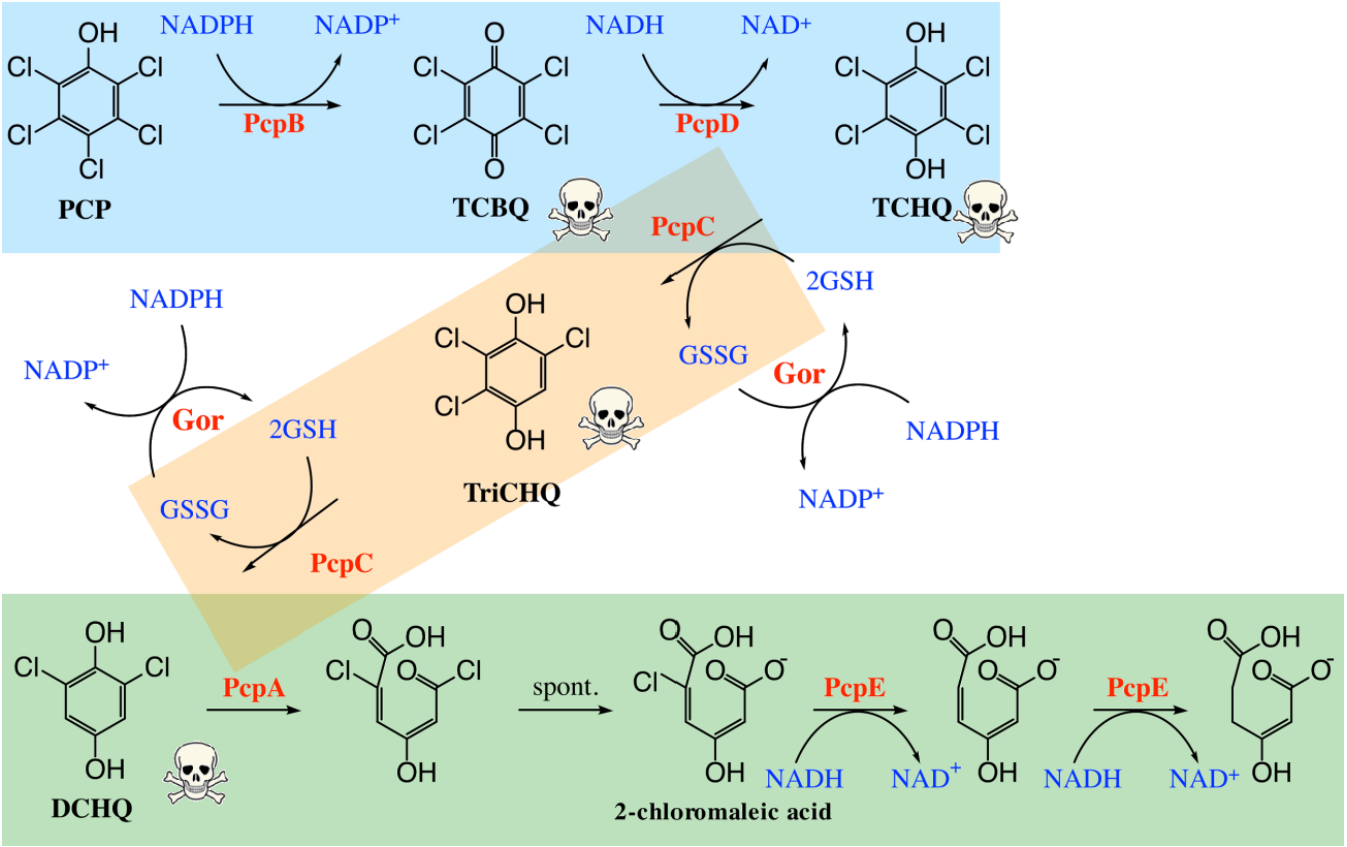

Figure 1B. PCP degradation pathway.

Some of the most important questions in biochemistry have to do with how enzymes (proteins that catalyze chemical reactions) interact with their substrates. PCP and some of the intermediates in this degradation pathway are toxic to the bacteria, decreasing the bacteria's capacity to fully degrade the substrates. To improve *S. chlorophenolicum's* ability to degrade PCP we need to think about how to improve flux through the pathway by making the enzymes quicker and more efficient at converting substrate to product, particularly when the substrates are toxic to the bacterium.

To meet our goal of improving PCP degradation while limiting substrate toxicity, we will first consider the concentrations of all the reaction intermediates within the bacteria by mass spectrometry. Next, we will consider data gathered by purifying the PCP degradation enzymes and using biochemical assays to measure their kinetics. These data will help us determine the rate limiting step within this pathway.

Questions

1. Based on the approximate cytoplasmic concentrations of the metabolites listed below in Table 1B, where do you think the limiting step is most likely to occur in this pathway and why?

   Table 1B. Concentrations of PCP and metabolites in cells treated with 670 µM PCP. (Modified from McCarthy *et al.*, 1997.)

Metabolite	*Approximate Cytoplasmic Concentration*
PCP	120 µM
TCBQ	0.01 µM
TCHQ	2 µM
TriCHQ	5 µM
DCHQ	2 µM
2-chloromaleic acid	1 µM

One way to compare the catalytic rates of enzymes is through their specificity constants (k_{cat}/K_M). The specificity constant takes into account k_{cat}, the number of substrate molecules each enzyme converts to product per unit time, and K_M, an inverse measure of the substrates affinity for the enzyme (lower K_M values indicate that the substrate has a strong likelihood of interacting with the enzyme).

2. The rate of a reaction at low substrate concentrations can be calculated using the specificity constant and current concentrations of enzyme and substrate, as described in the equation below where $[E_T]$ is total enzyme concentration and [S] is the concentration of substrate (from the previous table):

$$\text{Velocity} = k_{cat}\,[E_T]\,[S] / K_M$$

   Using Figure 1B and Table 1B, fill out the empty boxes in Table 2B below. Assume all enzyme concentrations are 10 µM.

   Table 2B. Catalytic rates of enzymes.

Enzyme	*k_{cat} (s^{-1})*	*K_M (µM)*	*k_{cat}/K_M ($\mu M^{-1}s^{-1}$)*	*Velocity (µM/s)*
PcpB	0.024	1		
PcpD	16.3	1.4	16.3 s^{-1} / 1.4 µM = 11.6 $\mu M^{-1}s^{-1}$	11.6 $\mu M^{-1}s^{-1}$ *10 µM * 0.01 µM = 1.16 µM/s
PcpA	3.05	3.2		
PcpE	1.2	30	1.2 s^{-1} / 30 µM = 0.04 $\mu M^{-1}s^{-1}$	0.04 $\mu M^{-1}s^{-1}$ *10 µM * 1 µM = 0.4 µM/s

   According to the calculated rates (last column above), which reaction is proceeding at the slowest rate?

3. Compare your answers to Questions 1 and 2. Do they agree?

4. The specificity constant only describes the reaction of an enzyme and its substrate at low substrate concentrations. When an enzyme is saturated with substrate, the substrate concentration and affinity no longer matter because we assume that all enzymes are converting substrate as fast as possible. Here, the catalytic rate can be calculated by:

$$\text{Velocity} = k_{cat} * [E_T]$$

   Assuming that PcpB is saturated, calculate its catalytic rate.

5. What factors impact flux through a metabolic pathway? Use the equations above to help you.

Enzymes must grab substrate, change it to product, and release the product in each enzymatic cycle. Experiments have shown that PcpB binds and converts substrate to product rapidly, but that the overall kinetic rate of the enzyme is very slow, $k_{cat} = 0.024\ s^{-1}$. These data indicate that release of TCBQ from PcpB must be the rate-limiting step (Rudolph *et al.*, 2014).

From the *National Geographic* article (Zimmer, 2013) you read for homework, you learned that *S. chlorophenolicum* avoids the release of the toxic intermediate TCBQ by retaining it in the active site of PcpB. The product is not released until PcpD bumps into PcpB and converts the retained TCBQ to TCHQ.

6. Considering that the formation of TCBQ is rapid, but physical interaction of two enzymes is necessary before TCBQ is reduced to TCHQ and released, propose a genetic modification to your bacteria that will increase the likelihood of PcpD encountering PcpB/TCBQ and making this key conversion (without releasing TCBQ).

7. Design an experiment to test your strain's ability to degrade PCP. (Think about what you need to measure to test this and important controls to have.)

8. You have improved the efficiency of the rate limiting step, but now your strain of *S. chlorophenolicum* has a much lower growth rate in the presence of PCP. Suspecting toxicity of PCP metabolism intermediates, you performed lethal dose curves with each of the metabolites (note that the dose is in log scale). Use Figure 2B below to complete Table 3B below of approximate LD_{50} values. Remember, an LD_{50} is the concentration of a toxin that results in 50% cell death.

Table 3B. LD_{50} values.

Metabolite	*LD_{50} (µM)*
TCBQ	
TCHQ	
TriCHQ	2.5 µM
DCHQ	4 µM
2-chloromaleic acid	> 100 µM

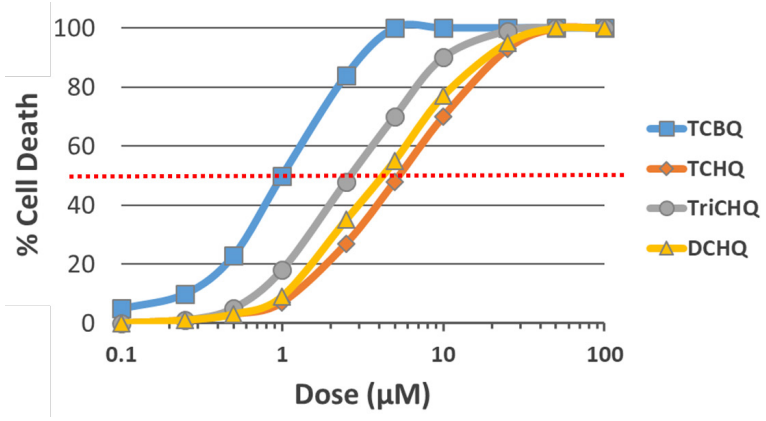

Figure 2B. Dose response curve.

9. You decide to measure the intracellular concentrations of intermediates in your new bacterial strain to see what might be contributing to cell death (Table 4B below). What do you hypothesize is causing your cells to die now and what would be your next approach for improving the system?

Table 4B. Concentrations of PCP and metabolites following genetic modification.

Metabolite	*Approximate Cytoplasmic Concentration*
PCP	10 µM
TCBQ	0.01 µM
TCHQ	50 µM
TriCHQ	5 µM
DCHQ	2 µM
2-chloromaleic acid	1 µM

For Next Meeting

Be ready to discuss the following questions at your next meeting (no written response necessary):

- During the degradation of PCP, which intermediates are toxic?
- What is the rate-limiting step of degradation? Why is this step so slow?
- What are some strategies for degradation of toxic compounds in which the intermediates are even more toxic than the original compound?
- Is there an evolutionary benefit of having a slow first enzyme in the pathway?

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Yellow Team: Metabolites and Growth

Bioremediation is defined as the use of microorganisms to remove pollutants, such as pentachlorophenol (PCP), from the environment. The *National Geographic* article "Mediocre Poison Eaters and the Imperfection of Evolution" (Zimmer, 2013) describes the inefficiency of PCP degradation (Figure 1ʏ), and it is clear that this metabolic pathway has not been optimized in nature to degrade PCP. Our goal as scientists is to create a strain of *Sphingobium chlorophenolicum* that can more efficiently break down PCP for use in bioremediation applications.

There are three interconnected considerations when engineering your improved strain:

1. Improving flux through the pathway to decrease exposure to toxic intermediates.
2. Balancing metabolites so that PCP degradation can happen alongside normal metabolism.
3. Decreasing the potential for PcpA inhibition through enzyme inhibition.

Yellow team goal: Improve the growth rate of *S. chlorophenolicum* by considering key metabolite consumption and how these metabolites are consumed or produced during normal bacterial growth.

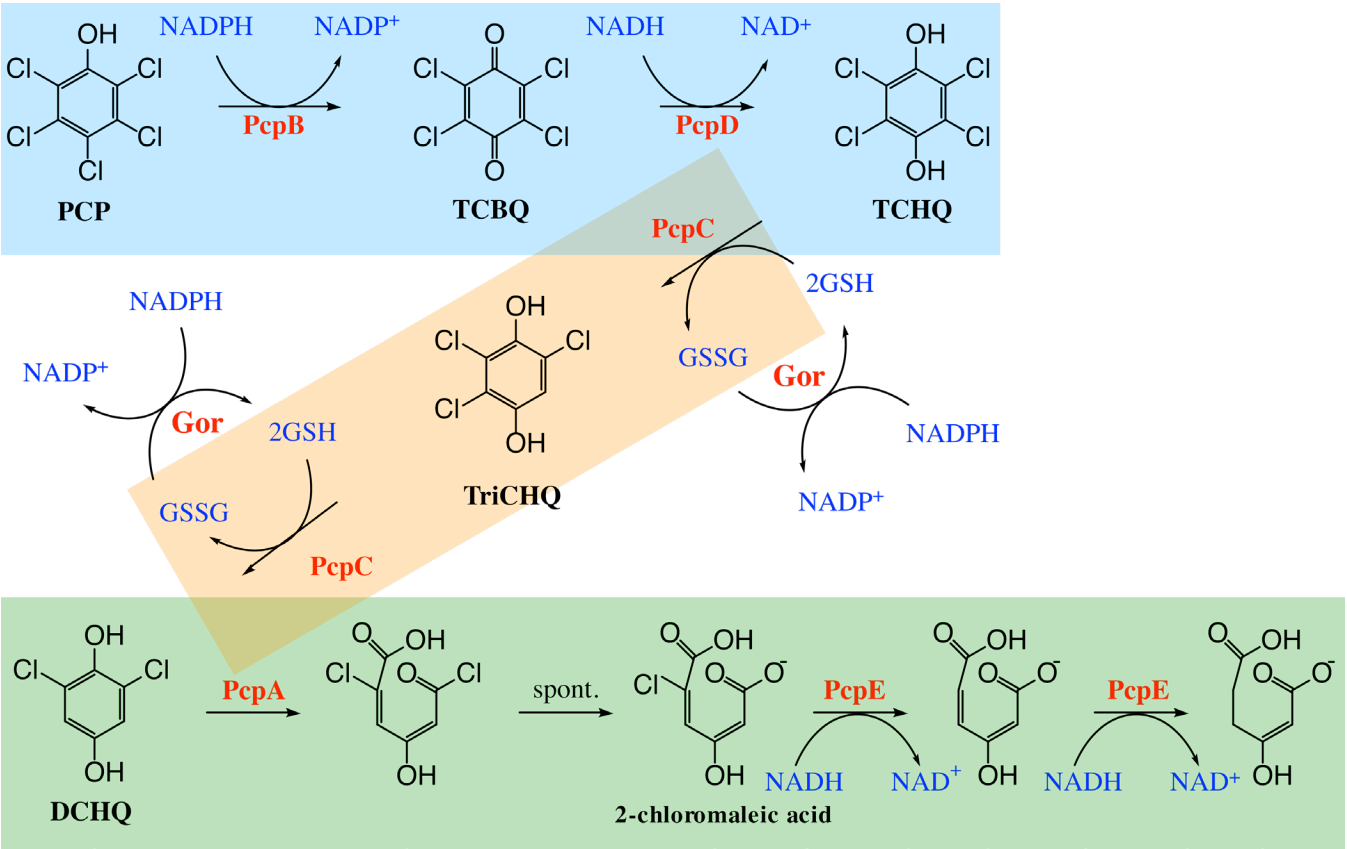

Figure 1ʏ. PCP degradation pathway.

To assess the general health and energy state of the cell during PCP degradation, we use mass spectrometry to compare the metabolite concentrations between strains grown in PCP to those grown in a control carbon source in the absence of PCP. Mass spectrometry identifies compounds based on their molecular mass and fragmentation patterns. An example spectra is shown below (Figure 2Y) for the amino acid L-cysteine.

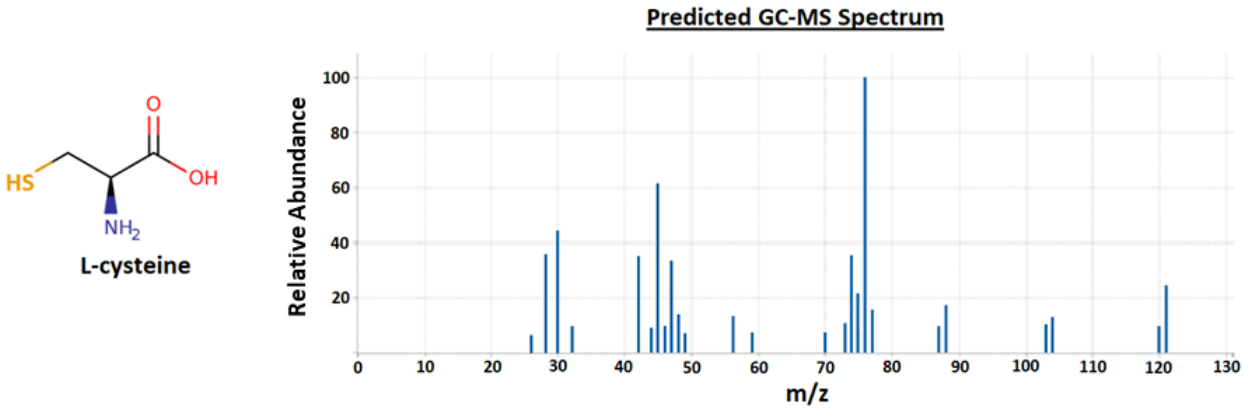

Figure 2Y. L-cysteine GC-MS spectrum. **From: The Human Metabolome Database**

The following metabolomics data was collected via mass spectrometry from three flasks of *S. chlorophenolicum* fed PCP, glucose, or both as their carbon source and grown for one week (Figure 3Y). Samples containing PCP (PCP or PCP + glucose) are normalized to the glucose control and presented as the $\log_2$ fold change. Log_2 fold change is a helpful way to visualize nutrient changes because it converts decreases in fold change values (lower in the PCP condition vs. glucose) to negative numbers that are easy to compare between conditions.

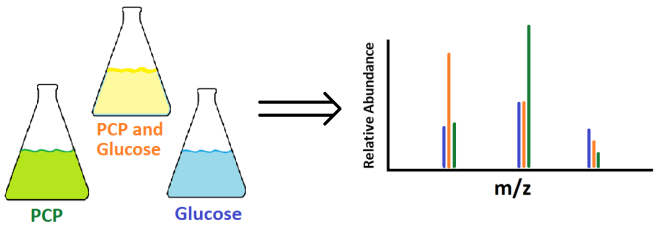

Figure 3Y. Metabolomics experiment diagram.

In Table 1Y below, if there are 100 counts of GSH in the glucose condition compared to 50 counts in the PCP condition, this represents a fold change of 0.5 because there are half as many molecules in the PCP condition. This is a two-fold decrease, represented in Table 1Y as $\log_2(0.5) = -1$ (see Figure 4Y).

Table 1Y. Log_2 fold change.

	Log_2 Fold Change in Counts (normalized to glucose control)	
Metabolite	*PCP*	*PCP + Glucose*
Glucose	−10	0
Glutathione (GSH)	−1	0
Glutathione disulfide (GSSG)	9	0.8
Adenosine triphosphate (ATP)	−0.2	0
Adenosine diphosphate (ADP)	0.75	0
NAD^+	0.03	0
NADH	−0.85	0
$NADP^+$	0.74	0.32
NADPH	−1	−0.26

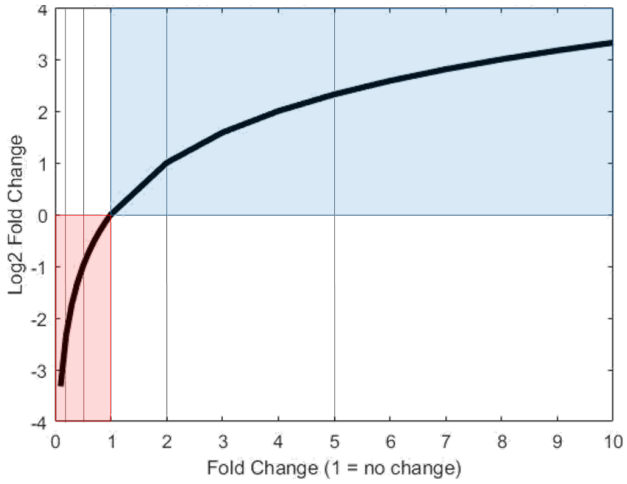

Figure 4Y. Relationship between fold change and $\log_2$ fold change.

Questions

1. In Table 1Y, draw a star next to all the metabolites that change at least two-fold when grown in the presence of PCP. Are these bacteria limited for any essential cofactors or metabolites?

2. Go back to the pathway diagram (Figure 1Y) and highlight metabolites that are limited in PCP conditions. What quantities of these metabolites are consumed during the degradation of one molecule of PCP?

3. We can also measure metabolite concentrations over time. Figure 5Y is a set of three graphs showing bacterial growth and metabolite ratios over time with PCP addition. Explain the graphs in your own words, paying particular attention to the timing of events and where each metabolite is used within the PCP degradation pathway.
   (a)

   (b)

   (c)

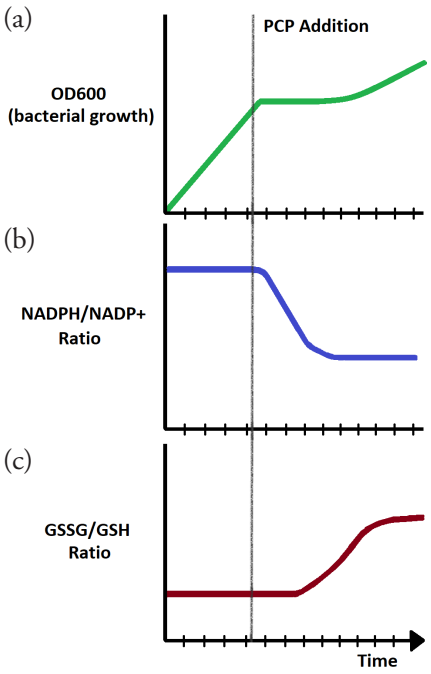

Figure 5Y. Graphs for Question 3.

4. Bacteria undergo depletion of NADPH in the process of breaking down PCP. Considering your knowledge of metabolism and the pathways shown in Figure 6Y, what is one way you could use metabolic engineering to facilitate regeneration of NADPH?

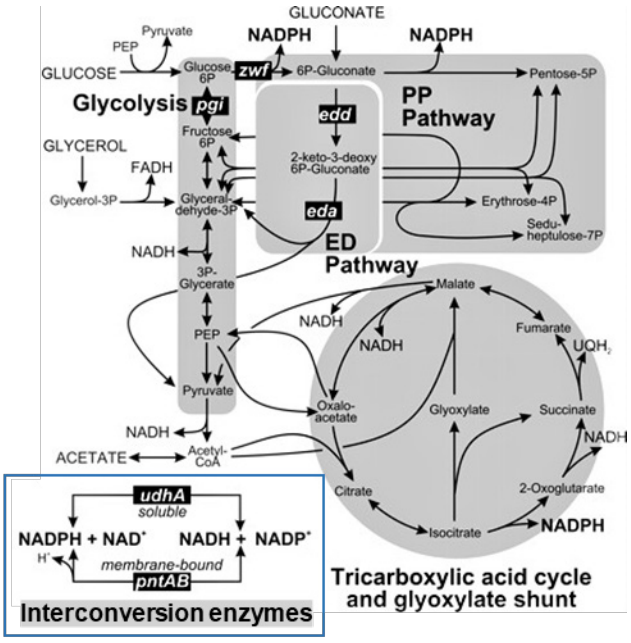

Figure 6Y. Pathways for Question 4. Modified from Sauer *et al.*, 2004.

5. Design an experiment to test the growth of your bacteria and balance of key metabolites if you make the genetic modification identified above. Draw your expected results on the graph in Figure 7Y.

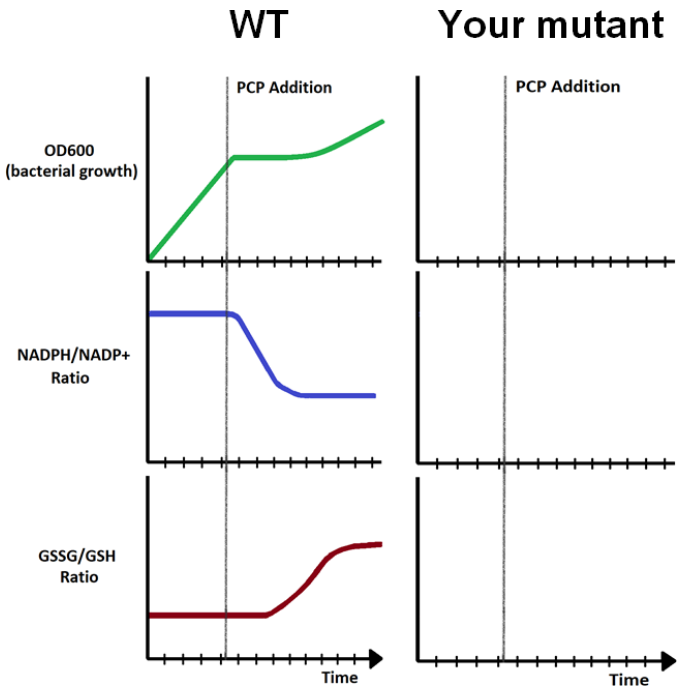

Figure 7Y. Predicted graphs for Question 5.

6. Pretend you added more glucose to replenish the NADPH in your bacteria rather than performing any genetic modifications. You get the experimental data in Figure 8Y. Explain the new data.

7. Adding glucose seems to diminish a lot of the detrimental growth effects of PCP addition. What else do you need to test after adding glucose to ensure that this modification is beneficial to the ultimate goal of degrading PCP?

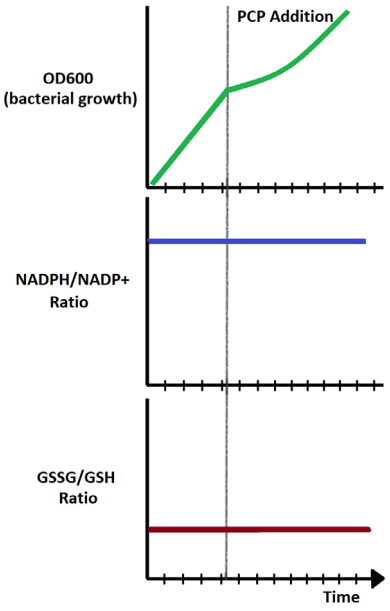

Figure 8Y. Data for Question 6.

For Next Meeting

Be ready to discuss the following questions at your next meeting (no written response necessary):

- Why is it important to consider the balance of key metabolites when metabolically engineering an organism?
- Which metabolites are particularly important for the degradation of PCP?
- Why is it important to maintain the selective pressure in which *S. chlorophenolicum* evolved?

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Green Team: Competing Pathways and "Moonlighting" Enzymes

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Green team goal: Use evolutionary knowledge of enzyme activity to avoid interactions with competing substrates and increase flux through the pathway.

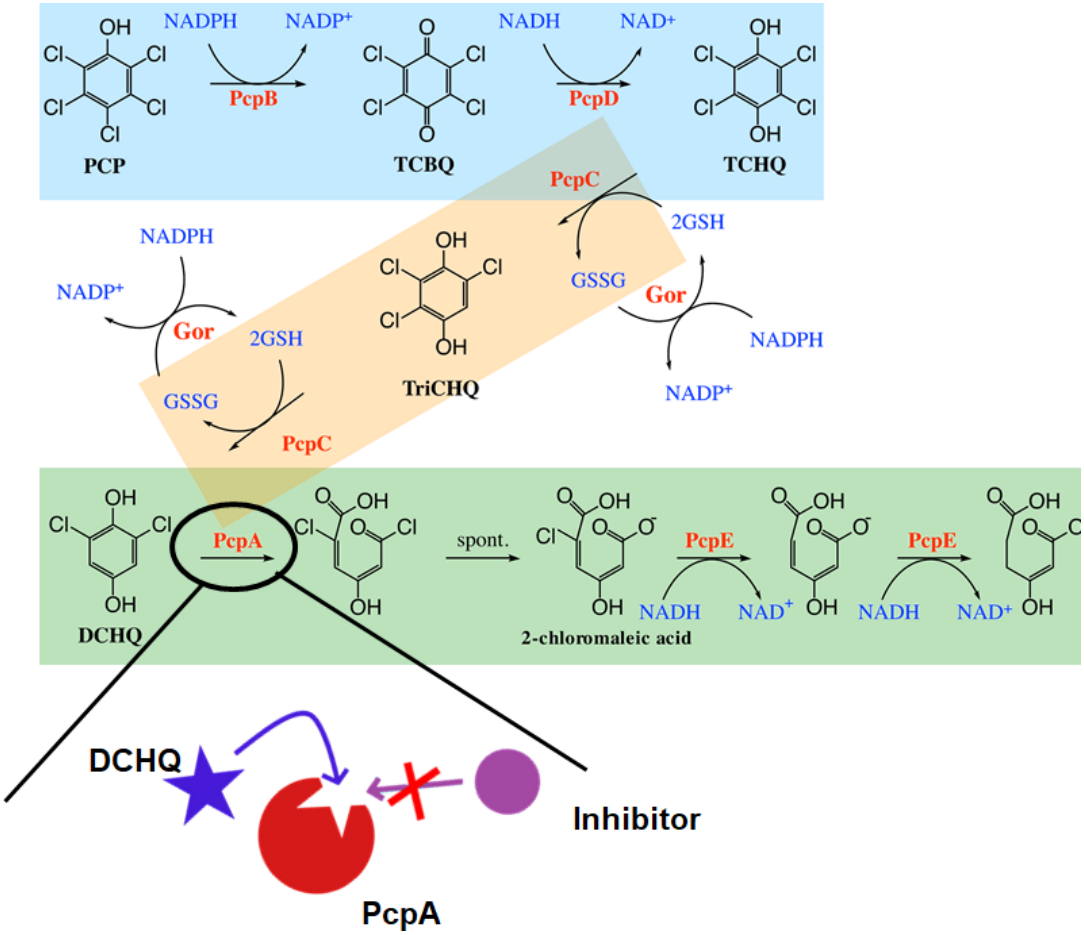


Figure 1G. PCP degradation pathway.

While several enzymes in the PCP degradation pathway were acquired through horizontal gene transfer, the enzyme that cleaves the aromatic ring, PcpA, already existed in *Sphingobium chlorophenolicum's* bacterial genome.

To better understand the role that PcpA plays in breaking down PCP, it helps to find enzymes with related structure or sequence. Below (Figure 2G) are the partial amino acid alignments (60 amino acids) of four bacterial enzymes that are similar to PcpA.

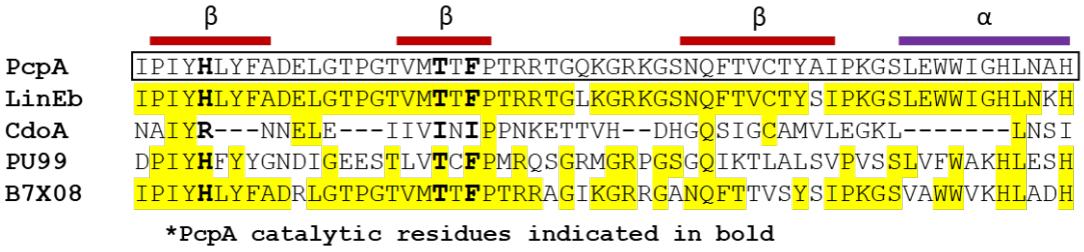


Figure 2G. Partial amino acid alignments of PcpA and four bacterial enzymes. *Credits:* Sequences from The UniProt Consortium, https://www.uniprot.org; alignment performed through Clustal Omega, https://www.ebi.ac.uk/Tools/msa/clustalo/.

Questions

1. Figure 2G depicts the sequence alignment of several enzymes with a section of the PcpA protein. Amino acids that align with PcpA are highlighted. Using that information, fill in Table 1G below with percentage sequence match.

Table 1G. Percentage sequence match.

	PcpA	*LinEb*	*CdoA*	*PU99*	*B7X08*
PcpA	100				
LinEb		100	13	42	77
CdoA		13	100	17	10
PU99		42	17	100	37
B7X08		77	10	37	100

2. To visualize how these enzymes are related evolutionarily, draw a phylogenetic tree (Figure 3G) demonstrating the evolutionary relationship between these five enzymes.

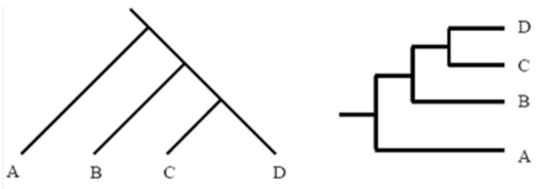
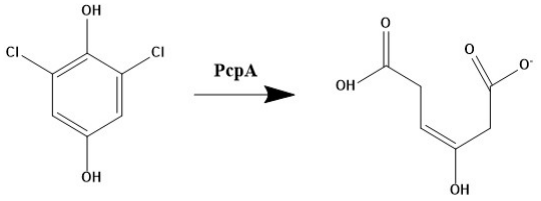
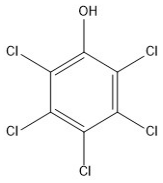
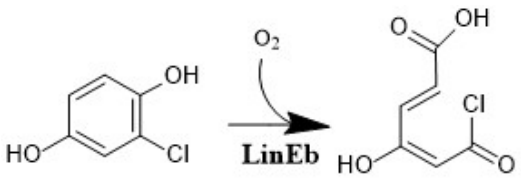
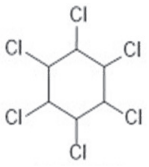
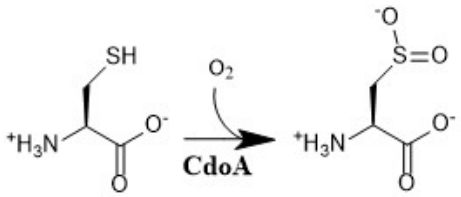
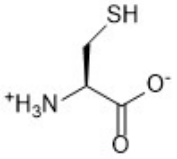
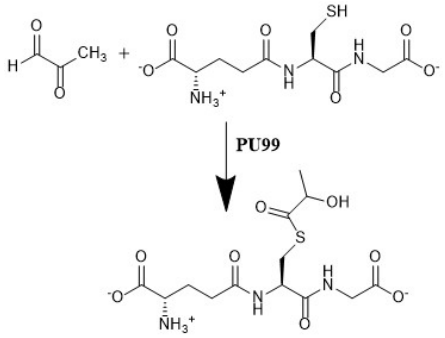
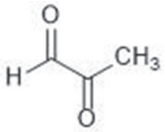
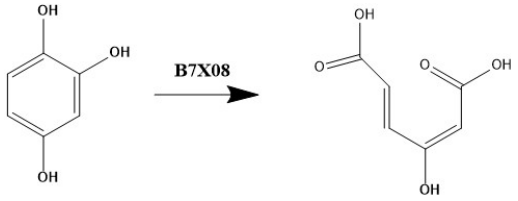
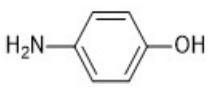


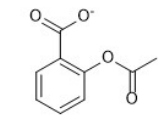
Figure 3G. Examples of phylogenetic trees.

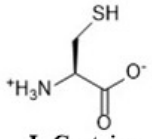
3. Based on the sequence alignment and enzyme table below (Table 2G), what is a potential evolutionary role of PcpA (long before humans began synthesizing new chemical compounds)?

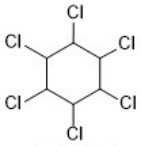
Table 2G. Enzymes.

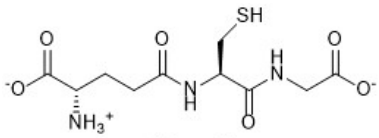
Name	*Organism*	*Reaction catalyzed*	*Ecological or Metabolic Role*
PcpA	*Sphingobium chlorophenolicum*		Break down PCP (toxic chemical in nature) 
LinEb	*Sphingobium japonicum*		Break down Lindane (common insecticide) 
CdoA	*Bacillus subtilis*		Cysteine catabolism 
PU99	*Pseudomonas putida*		Detoxification of methylglyoxal via glutathione 
B7X08	*Acidocella*		Break down phenol rings, from metabolites or environmental chemicals 

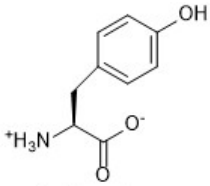
4. Which of the following are potential competitive substrates of PcpA, and why do you think so?


Acetylsalicylic acid (Aspirin)


L-Cysteine


Lindane (insecticide)


Glutathione


L-Tyrosine

5. Many molecular interactions play a role in enzymes recognizing their appropriate substrate including size and charge. What are two other factors that play a role in enzymes recognizing the proper substrate?

6. How can you use this information to modify PcpA to avoid competing substrates? (See Figure 6G for hint.)

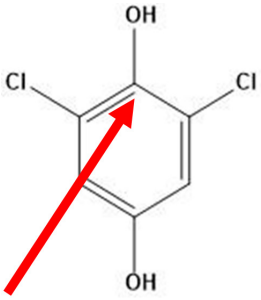


Figure 6G. The red arrow shows where PcpA cleaves DCHQ.

7. You made your mutation to PcpA and produced a new enzyme, PcpAX. How could you test your new PcpAX enzyme and demonstrate that it has increased specificity for DCHQ? How could you demonstrate that its efficiency has not decreased? (Make sure to include the proper experimental controls.)

8. Suppose L-tyrosine has been identified as an alternative substrate for PcpA. Below is a Lineweaver-Burk plot for PcpA/DCHQ in the presence and absence of L-tyrosine. In the right plot, draw your predicted curves for PcpAX/DCHQ in the presence and absence of L-tyrosine.

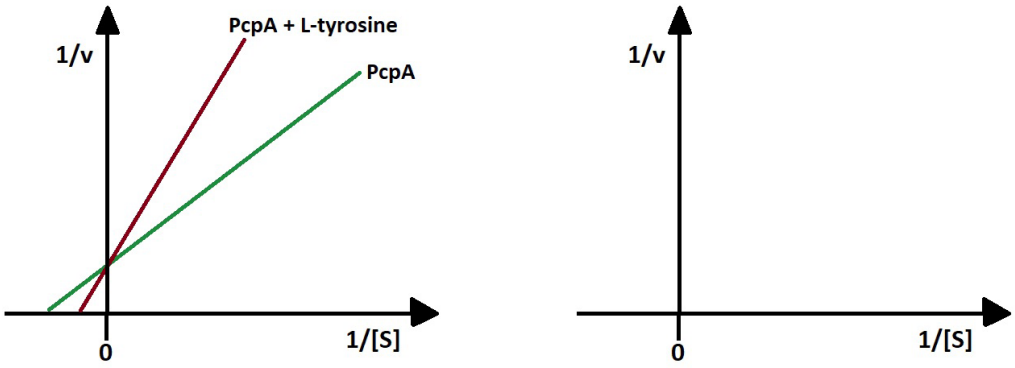


Figure 7c. Lineweaver-Burk plot for PcpA/DCHQ.

9. Why did you draw the lines as you did?

10. What type of inhibition does L-tyrosine exhibit on PcpA? How can you distinguish this type of inhibition from other forms?

11. Why do you think PcpA has retained its ability to interact with L-tyrosine rather than evolving to become more specific for DCHQ?

Biological engineers have developed new ways to create bacterial strains with desired traits in the laboratory. Adaptive laboratory evolution uses bacteria growth and serial dilutions under certain selection conditions to select for a desired trait over many many generations. Directed evolution involves making genetic manipulation via error-prone or directed mutation PCR, transfecting these bacteria with new messy genes, and selecting for advantageous mutations in these populations.

12. How could you use directed evolution in the laboratory to push evolution in the favor of greater DCHQ specificity?

13. Imagine PcpA is the only enzyme that can break down L-tyrosine in your cells and this ability is lost after 15 rounds of directed evolution. What are likely metabolic consequences?

For Next Meeting

Be ready to discuss the following questions at your next meeting (no written response necessary):

- What information can you gain from evolutionary analysis of proteins?
- Which type of compounds are most likely to be competitive inhibitors of the PCP degradation pathway?
- How can you modify proteins to change their substrate specificity?
- How can you apply the idea of competing substrates to aid in metabolic engineering?

Jigsaw Worksheet

Questions

1. Explain to your new partners which aspect of PCP metabolism you focused on, ways that you would make the pathway more efficient, and potential caveats to your proposed method. Use the jigsaw questions on your group sheets to guide you.

2. In groups, draw on the pathway below (Figure 1) your plan to optimize the system. Keep in mind growth rates, degradation of PCP and its toxic intermediates, and competing pathways.

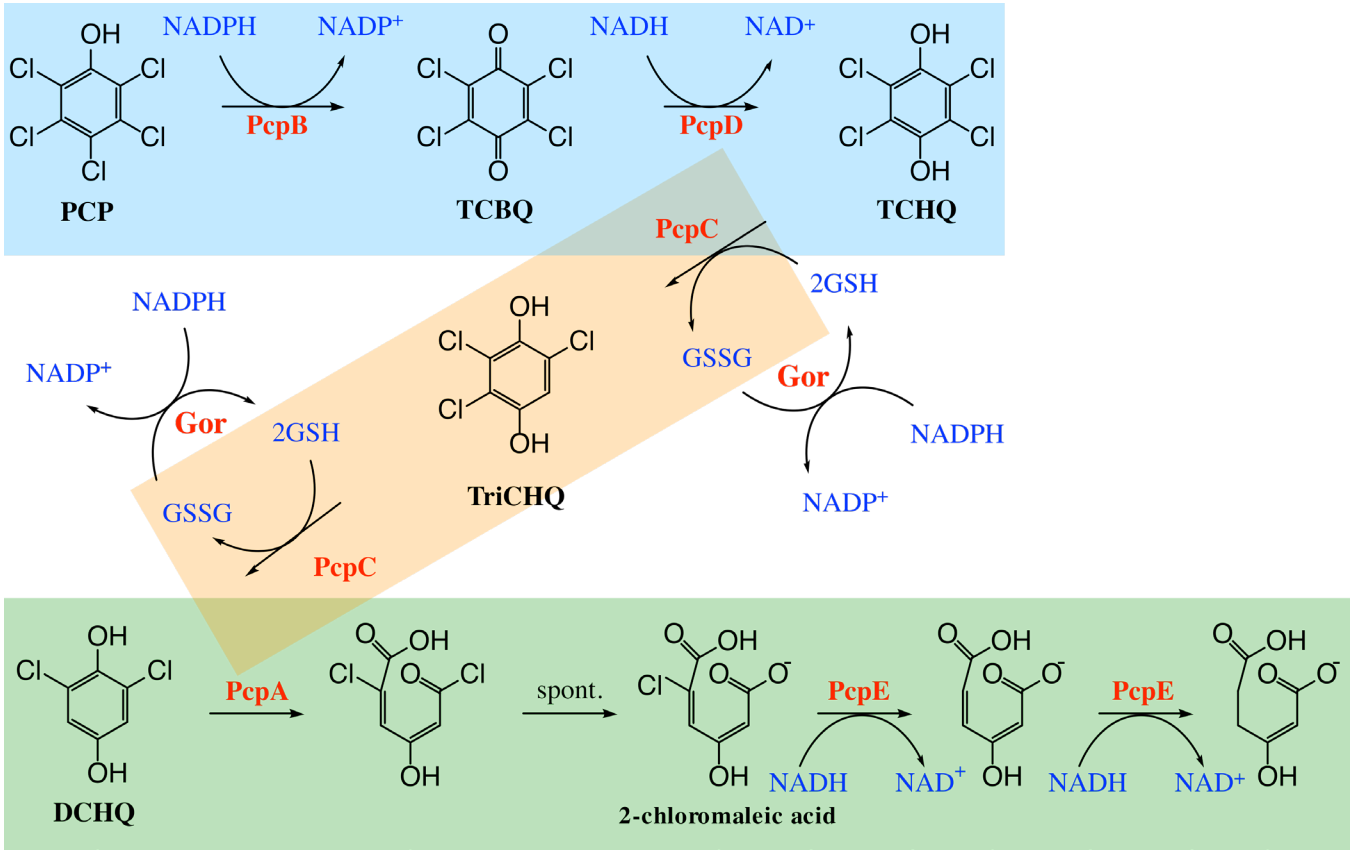


Figure 1. PCP degradation pathway.

3. Design an experiment to test your new strain.

   Note: If you propose multiple modifications to optimize the bacteria, the modifications would likely be made step-wise in the laboratory, so you can propose an experiment to test just one of the modifications below.

   Hypothesis:

   Methods:

   Necessary controls:

   Expected results:

4. Think deeper:
    a. You are hired to develop a new antibiotic that does not kill a specific class of important gut microbes. What are three considerations you need to keep in mind when designing this drug?

    b. A fertilizer company contacted you to say they are worried their insecticide is toxic to animals and humans. This insecticide is entering the Denver water source, and they want your help finding a way to degrade or dispose of the chemical before the city gets sick or spends millions of dollars on water treatment. What are two suggestions you give them?

Reflection

- What is the most interesting thing you learned from this activity?

- What information would have been helpful in completing this activity?