Project 90170
Genetic Engineering of Plants to Improve Phytoremediation of Chlorinated Hydrocarbons in Groundwater

Stuart E. Strand
University of Washington

RESULTS TO DATE:

I. Mechanism of halogenated hydrocarbon oxidation We are using poplar culture cells to determine the pathway of TCE metabolism. In our earlier work, we found that trichloroethanol (TCEOH) is a major early intermediate. Our studies this year have focused on the steps that follow this toxic intermediate. We did several experiments to track the disappearance of TCEOH after the cells were removed from TCE. We could conclude that TCEOH is not an end-product but is rapidly degraded. Six flasks of poplar liquid suspension cells were exposed to a level of 50 µg/ml TCE for three days. Three of the cultures were subjected to MTBE extractions to quantify the levels of TCEOH produced. The cells of the remaining three cultures were then pelleted and resuspended in fresh medium. After three more days, these were also subjected to MTBE extractions. The samples were analyzed by GC-ECD. After the three days of further metabolism, an average of 91% of the trichloroethanol was gone. When similar experiments were done with intact plants and both free and conjugated TCEOH were quantified, a similar rapid decline in both forms was seen (Shang, 2001). Therefore, it seems probable that similar mechanisms are taking place in both poplar suspension cells and whole poplar plants, so we continued to do our studies with the suspension cells. Metabolism of trichloroethanol may go through trichloroacetic acid (TCAA) prior to dehalogenation. To test this possibility, we exposed cells to TCE and analyzed for TCAA over time. The cultures were analyzed after 4, 5, 6, and 14 days from TCE exposure. We did not detect any significant amount of TCAA above the background in undosed cells. To determine if trichloroethanol itself is directly dehalogenated, we analyzed TCE-exposed cells for the presence of dichloroethanol. Undosed cells did not have any of the DCEOH peak but TCE-dosed cells that produced the highest levels of trichloroethanol did have a small DCEOH peak. Cultures that did not produce high levels of TCEOH did not have the DCEOH peak. This result repeated in two independent experiments. We decided to expose cells directly to TCEOH and look for DCEOH in the cell extracts. After one week of exposure, the culture cells produced consistent levels of DCEOH of approximately 0.02% of the TCEOH dose. However, when we did a control reaction with no cells, DCEOH was present, indicating that the TCEOH degrades in the absence of cells. We are currently conducting the same experiments with newly-purchased chemicals and in darkness (by wrapping the culture flasks in foil). We have had success using tribromoethanol as a surrogate for trichloroethanol in studying the dehalogenation reaction in poplar cells. We had previously shown that tribromoethanol is steadily metabolized over time in poplar culture cells, producing free bromide ion. TBEOH-dosed dead cells and no cell controls did not have any bromide ion production. We are currently using this system to test P450 inhibitors to determine if dehalogenation of TBEOH is through this mechanism. We have recently purchased tribromoethylene as a more easily monitored surrogate for TCE. We will conduct mass balance experiments to determine what percentage of the bromide is released from tribromoethylene.

II. Plant Genes Involved in Halogenated Hydrocarbon Oxidation Previously, we had demonstrated that Arabidopsis thaliana culture cells can metabolize TCE to trichloroethanol. Therefore, this plant would be suitable for cloning the genes involved in TCE metabolism. We purchased the cDNA library of A. thaliana Colombia and amplified the phagemids in E. coli. We tested a variety of pollutants for the ability to kill the E. coli strain such that we can screen for survival. Our results indicated that the primary chemicals (TCE, CT, and EDB) do not kill the cells, it is rather the early metabolites of these chemicals that cause harm. Therefore, we switched to screening the library on trichloroethanol which does kill the cells. We have screened thousands of cells without seeing any true survivors with the cDNA library. Next, we are going to test dichloroethanol since metabolism of this substrate may lead

to survival more readily than metabolism of trichloroethanol. We found a supplier of this chemical (ChemServices) and have placed an order.

III. Testing of CYP2E1 Transgenic Poplars We had previously published the profound effects of overexpressing mammalian cytochrome P450 2E1 in tobacco plants (Doty2000). When we tried using the same constructs to transform poplar plants, however, the enhancement of TCE metabolism was much less pronounced. We determined that the problem was at the level of expression of the cDNA. We constructed a different plasmid using the CaMV35S promoter that has been shown to work very well in poplar. This plasmid (pSLD50-6) was introduced into Agrobacterium tumefaciens and Agrobacterium rhizogenes strains and was used to transform the aspen hybrid, 717-1B4 from the INRA (France). Numerous transgenic lines were propagated. The transgenic hairy root lines from A. rhizogenes have not grown sufficiently for metabolism experiments to be performed, therefore, we are focusing our attention on the transgenic plants generated from A. tumefaciens. Since aspens are notoriously difficult to root from cuttings, we had to adapt our metabolism assay. Rather than grow rooted plants in flasks with the foliage exposed to air, we place small cuttings in 40-ml VOA vials containing 10 ml of plant nutrient solution, and dose with TCE through a mini-nert valve. Each transgenic line was tested in duplicate and compared with plants that were transformed with the null vector. After one week of exposure to TCE, the plants were ground to a powder in liquid nitrogen with a mortar and pestle, and extracted using our standard MTBE protocol. Trichloroethanol, the first major metabolite of TCE, was quantified using gas chromatography. The amount of trichloroethanol produced per gram of plant tissue was compared, and the results were that most of the transgenic plants did show enhanced metabolism of TCE. The best transgenic plants which we have screened so far have levels of 6, 13, 21, 31, and 33 fold above that of the average of the vector control plants. These plants are being propagated for further study while we continue to screen more of the transgenic plants.

IV. Methods for comparison of wild-type and transgenic poplar metabolism of chlorinated hydrocarbons Laboratory and greenhouse studies are underway to compare CT and TCE degradation by wild-type and transgenic poplar and to select the best transgenic clones prior to their testing at full-scale at the test-bed site. Poplars were grown in a flow-through system consisting of glass columns. Profiles of solvent concentration vs. depth in the root zone indicate that the transgenic poplars removed 6% more TCE and 20% more CT from influent water than the wild-types. TCE degradation was found to be correlated with oxygen levels in the enclosed root zone, an observation that may explain previously reported differences between laboratory and field uptake and fate of TCE by plants. These experiments are continuing with a modified apparatus to provide oxygen levels more representative of field conditions. Following greenhouse screening we will plant the best transgenic poplar in our test bed site operated in conjunction with an NIEHS grant and in collaboration with Occidental Chemical Co. Studies of the uptake and degradation of carbon tetrachloride (CT) by wild-type poplar have been completed at the test bed side. The fate of carbon tetrachloride (CT) during phytoremediation with poplar was assessed by examining the transpiration of CT from leaves, diffusion from soil, tree trunks and surface roots, and accumulation of chloride ion in soil and plant tissues. Feed water containing 12-15 mg/L CT was added to the field test beds planted with poplar and over 99% of the CT was removed. No significant amount of CT was transpired or diffused into the air and no significant amount of CT-chlorine accumulated in the tree tissues. Chloride ion accumulated in the soil accounted for all of the CT-chlorine removed. When soils from the root zones were compared to unvegetated soils, microbial mineralization of CT was not enhanced in soils from the root zones compared to unvegetated soils. Thus, we conclude that uptake and dechlorination of CT by plant tissues is likely the primary mechanism for phytoremediation by poplar. A paper describing these results is in press in Environ. Sci. Technol. We have also completed experiments aimed at resolving controversies surrounding the role of rhizosphere soil microorganisms in poplar phytoremediation. Previous mass balance studies of phytoremediation of chlorinated hydrocarbons have shown that more than 95% of applied carbon tetrachloride (CT), trichloroethylene (TCE), and perchloroethylene (PCE) was removed in poplar tree plantings. Laboratory studies using radiolabeled compounds demonstrated that degradation of CT in soils from poplar root zones was not enhanced compared to unvegetated soils under either anaerobic or aerobic conditions. TCE and PCE degradation was not enhanced under aerobic conditions typical of poplar planted soils; but under anaerobic conditions, TCE and PCE

transformations were enhanced in soils associated with poplar roots compared to unvegetated soils. 13C from isotopically-labeled CT and TCE in root chambers incubated in situ was enriched in root tissues, but not in the soil. These results suggest that microbial activity during phytoremediation of CT, TCE and PCE is not enhanced in aerobic root zone soils compared to unvegetated soils. This paper has been submitted to Water Research. In the summer of 2004 we completed a mass balance season with poplar exposed to PCE, including trunk and soil diffusion pathways. Upon completion of the PCE analysis we should be able to compare PCE, TCE and CT uptake.