(사)한국 지하수토양환경학회
추계학술대회논문집
2000년11월17일 포항공대 환경공학동

Enhanced Phytoremediation of Trichloroethylene-Contaminated Soil by Poplar-Colonizing Recombinants

심 호 재

한양대학교 토목 • 환경공학과
(E-mail: hojaeshim@usa.net)

ABSTRACT

Indigenous bacteria from poplar roots (*Populus canadensis* var. *eugenei*, 'Imperial Carolina') and Southern Californian shrub rhizospheres as well as two tree-colonizing *Rhizobium* strains (ATCC 10320 and 35645) were genetically engineered to express constitutively and stably toluene *o*-monooxygenase (TOM) from *Burkholderia cepacia* G4 by integrating the *tom* locus into the chromosome. The poplar and *Rhizobium* recombinants degraded trichloroethylene (TCE) at 0.8-2.1 nmol/min • mg protein (initial TCE concentration, 10 μM) and competitive against the unengineered hosts in wheat and barley rhizospheres for one month (colonization at 1 - 23 x 10^5 CFU/cm root). In addition, six of these recombinants colonized poplar roots stably and competitively with populations as high as 79 ± 12% of all rhizosphere bacteria after 28 days (0.2 - 31 x 10^5 CFU/cm root). Furthermore, five of the most-competitive poplar recombinants (e.g., Pb3-1 and Pb5-1 which were identified as *Pseudomonas* PsK) retained the ability to express TOM for 29 days as 100 ± 0% of the recombinants detected in the poplar rhizosphere had constitutive expression of TOM.

Key words: Enhanced phytoremediation, trichloroethylene, poplar, recombinants

I. INTRODUCTION

Plants are useful for bioremediation as it has been known for at least 70 years that some species accumulate toxic metals (23), and they have been shown to stimulate degradation of polycyclic aromatic hydrocarbons (4) and 2,5-dichlorobenzoate (6). In Kuwait, many crop species were shown to grow in

soil containing up to 10% crude oil by weight and to cleanse the rhizosphere of the crude (14). The term rhizosphere refers to the environment influenced by plant roots in which elevated bacterial activity is observed. Rhizoremediation (the degradation of recalcitrant pollutants by bacteria in the rhizosphere) is an attractive process since plant roots provide a large surface area for a significant population of bacteria and transport the root-colonizing, remediating microorganisms to pollutants 10 to 15 m deep in the soil. Root-colonizing bacteria genetically engineered to degrade pollutants should retain their competitive advantage in the rhizosphere compared with indigenous non-root-colonizing bacteria. In addition, horizontal transfer can be reduced by incorporating the biodegradation genes into the bacterial chromosome; once an area is remediated, harvesting the plants should remove the niche for the specific bacteria. Consequently, rhizoremediation appears to be an aesthetically pleasing, low-cost, minimal-maintenance, in situ treatment for pollutants in surface soils. Rhizoremediation has shown promise based on the use of wild-type bacteria in their native environments to degrade a variety of pollutants. Although employing indigenous microorganisms and plants to treat contaminated soils often requires the identification of suitable, preexising natural systems, bacteria known to colonize specific plants may be engineered to gain more control over the process.

For chlorinated aliphatics, plants and wild-type bacteria have been shown to increase trichloroethylene (TCE) degradation in the rhizosphere; for example, the legume (*Lespedeza cuneata*) converted 30% of [^{14}C]TCE to [^{14}C]CO_2 (3). Since World War II, TCE has been used as the standard solvent for degreasing metal parts and textiles, and demand for TCE may increase with U.S. Environmental Protection Agency approval of TCE as an alternative for chlorofluorocarbon 113 and methyl chloroform. TCE is a suspected carcinogen and is one of the most-common groundwater pollutants at hazardous waste sites (11), and this volatile organic compound is second only to trihalomethanes (i.e., bromodichloromethane, bromoform, and chloroform) as the most frequently detected compound in municipal groundwater supplies.

To take advantage of the plant-bacteria relationship to degrade chlorinated aliphatics, the genus *Populus* [which includes poplars, cottonwoods, and aspens (5)] was chosen for this work since these trees have many advantages for bioremediation. Poplars grow quite rapidly (3-5 m/yr) since foresters have crossed

them for years to maximize growth rates and yields. In addition, they have extended roots which can reach to the water table; therefore, they have the capacity to treat the saturated zone (5). A five-year-old tree can process over 53 gallons of water per day (5); hence, 53 gallons of water contaminated with TCE could pass through the rhizosphere and be treated by engineered bacteria.

It is attractive to combine genetically engineered bacteria with poplar trees since engineered bacteria in field trials frequently languish from low survivability in the absence of plants (18). However, the rhizosphere provides controlled conditions for the symbiotic growth of the engineered strains adapted for roots. Roots provide ideal attachment locations, steady redox conditions, and a steady food supply of exudates consisting of organic acids, enzymes, amino acids, and complex carbohydrates (4, 25); hence, engineered rhizobacteria have a niche to flourish [bacterial populations in the rhizosphere are two to three orders of magnitude higher than those in the outlying soil (4)]. Since high levels of phenolic compounds have been found in root exudates (8), these compounds also serve to induce bacterial dioxygenase remediation pathways (22). Along with providing the bacteria both nutrients and oxygen for chlorinated aliphatic degradation, the trees also transport the bacteria throughout the TCE-contaminated soil (from surface to aquifer), transport TCE-contaminated water to the rhizosphere, and aerate the soil.

Toluene *o*-monooxygenase (TOM) of *Burkholderia cepacia* G4 is encoded by six genes (*tomA012345*) (17) and is a three-component enzyme consisting of a 211 kDa hydroxylase with a catalytic oxygen-bridged binuclear iron center, a 40 kDa reductase, and a 10.4 kDa protein involved in electron transfer between the hydroxylase and reductase (13). TOM oxidizes TCE, all 3 dichloroethylenes, and vinyl chloride (17, 19), and TCE is degraded primarily to CO_2 and Cl^- *in vivo* (10, 12). Recent studies also indicate strains expressing TOM can degrade mixtures of these compounds (21).

To create a general cloning vector capable of integrating the genes for the TCE-degrading TOM enzyme into virtually any Gram-negative bacterial chromosome, the 11.3 kb DNA fragment containing kanamycin (Km) resistance and the constitutive IS*50* promoter of Tn*5* (17) fused to *tomA012345* was ligated into the suicide Tn*5* transposon plasmid pCNB4 (7) to form pLANT3 (26). pLANT3 does not replicate in non-λ *pir* lysogens; after conjugation, a single transposition event occurs to integrate the *tom* locus and Km resistance into the

chromosome. This method was used previously to form the wheat root-colonizing strain *Pseudomonas fluorescens* 2-79TOM (26) which continuously expressed active TOM at high levels (enzyme stable for up to 290 generations) and which degraded TCE in wheat rhizospheres (26).

In this study, to utilize the advantages of poplar trees and recombinant bacteria for TCE remediation, wild-type bacteria were isolated from the poplar tree rhizosphere, converted to TCE-degrading strains by integrating TOM into the chromosome, and evaluated for their ability to compete in the poplar tree rhizosphere and stably express TOM. This is the first report of the construction of tree-colonizing bacteria for bioremediation.

II. MATERIALS AND METHODS

1. Microorganisms

Uncharacterized poplar tree-colonizing bacteria (PM and Pb series, Table 1) were obtained by sonicating roots of hybrid poplar stems (*Populus canadensis* var. *eugenei*, 'Imperial Carolina', from Vans Pines Nursery, West Olive, MI and Segal Ranch, Grandview, WA) for 20 sec at 4 watts using a Sonic Dismembrator Model 60 (Fisher Scientific Company, Fair Lawn, NJ), plating the resulting solution on Luria-Bertani (LB) medium (16) and incubating the plates at 30 °C. The roots of two native shrubs from Crystal Cove, CA were similarly treated and two isolates (S and H) were obtained. The wheat colonizers *P. fluorescens* 2-79 and *P. fluorescens* 2-79TOM (26), which expresses TOM constitutively, were used along with two tree-colonizers obtained from the American Type Culture Collection (Rockville, MD): *Rhizobium* spp. ATCC 10320 (from black locust, *Robina pseudoacacia*) and ATCC 35645 (from Haole koa, *Leucaena leucocephala*).

2. Phylogenetic identification of organisms

The two most-competitive poplar recombinants, Pb3-1 and Pb5-1, were identified using 16S rDNA analysis. Bacterial genomic DNA was extracted using a Triton-prep method. In this procedure 5 mL of an overnight culture was mixed with 300 μL STET (8% sucrose, 5% Triton X-100, 50 mM tris-HCl pH 8.0, 50 mM EDTA pH 8.0), 20 μL 50 mg/mL lysozyme (Sigma, St. Louis, MO), and 10 μL 10 mg/mL RNase A (Sigma). This mixture was boiled for 1.5 minutes,

centrifuged for 15 minutes, and the supernatant extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) (Fisher Scientific). The DNA in the aqueous phase was precipitated using isopropanol and washed with 70% ethanol. After vacuum drying, the DNA was suspended in 200 μL water. Polymerase chain reaction (PCR) was performed on 5 μL of the genomic DNA in 50 μL reactions using standard cycling conditions (94 °C for 5 min, and 30 cycles at 94 °C for 1 min, 72 °C for 1 min, followed by 10 min at 72 °C). Two sets of universal 16S rDNA primers were synthesized by Gibco BRL (Gaithersburg, MD) (position on *E. coli* chromosome listed), HK12 (2) 5′-GAGTTTGATCCTGGCTCAG (10-28)/HK13 5′-TACCTTGTTACGACTT (1492-1507) and JCR15 (2) GCCAGCAGCCGCGGTA (517-532)/JCR14 (2) ACGGGCGGTGTGTAC (1392-1406). HK12/HK13 amplified the entire 16S rDNA region (1497 bp) while JCR14/JCR15 amplified 889 bp within the 16S rDNA. Each set of primers was used in triplicate PCR reactions for each isolate so that there was two-fold redundancy to avoid errors (two 16S rDNA regions and each region sequenced three times). The PCR products were purified with Qia-Quick PCR purification kit and the DNA was sequenced using an automated ABI automated sequencer.

3. Conjugation

Based on colony morphology, eight different hosts obtained from the LB plates containing the isolates from the poplar and native shrub roots which showed growth on glucose M9 minimal salts (16) but no growth on LB containing 50 μg/mL Km were conjugated with donor *Escherichia coli* S17-1 (λ*pir*)/pLANT3 containing *tomA012345* as described previously (26) to create PM2-1,2,3, PM4-1,2,3, Pb1-1, Pb2-1, Pb3-1, Pb5-1, S-16, and H-25 (Table 1). Two *Rhizobium* recombinants expressing TOM (10320D and 35645A) were similarly obtained after conjugating their corresponding hosts (*Rhizobium* spp. 10320 and 35645) with *E. coli* S17-1 (λ*pir*)/pLANT3. Only one transposition event occurs (7) with pLANT3 since the enzyme for transposition does not integrate along with *tomA012345*.

4. Rhizosphere competitiveness assays

The competitiveness of representative recombinants, including two poplar

recombinants (Pb3-1 and Pb5-1), a *Rhizobium* sp. recombinant (35645A), *P. fluorescens* 2-79TOM, and two shrub recombinants (S-16 and H-25), against their corresponding wild-type hosts was tested in rhizospheres of barley and wheat in sterile soil. Seeds of Moongold barley (Liberty Seed Company, New Philadelphia, OH) and Cavalier winter wheat (Stover Seed Co., Los Angeles, CA) were first sterilized with 95% ethanol for 10 sec, then with 3% hydrogen peroxide for 3 min, and germinated by incubation upon wet sponges for 2 - 5 days at room temperature in the dark. The sponges were sterilized by soaking in a 2.5% sodium hypochloride solution for 30 min, rinsed with distilled water, then individually autoclaved in a foil-covered beaker containing water (sponge not submerged).

Germinated seeds were inoculated with mixed cultures of recombinants and hosts by placing the seeds in an open Petri plate in a laminar flow hood. Recombinant and wild-type host strains were grown overnight in LB to a final absorbance at 600 nm (OD_{600}) of 1.4 - 1.6 (recombinant with 50 μg/mL Km). After centrifugation at 10,000 rpm for 5 min at 25 °C, both cell pellets were resuspended in 0.1 M potassium phosphate buffer (PPB, pH 7), then the OD was adjusted to have the same OD for both recombinant and host in 10 mL of 0.1 M PPB. The resulting solution was transferred to the seed-containing plate and allowed to dry for 4 hr. The seeds (10 - 15) were planted, using sterile forceps, in plastic pots containing 300 g of sterile soil and 50 mL of sterile tap water. The potted plants were placed 2 ft under 60-W Gro & Sho plant light bulbs (General Electric, Cleveland, OH), illuminated 16 hr each day, and watered with 25 mL sterile tap water every 48 hr.

The competitiveness of 15 of the recombinants against indigenous organisms present in non-sterile soil (Mansfield Center, CT) was determined in the poplar rhizosphere. Poplar stems were cut 6" long and grown hydroponically to form roots in 25% modified Hoagland's solution (per liter: 0.21 g KH_2PO_4, 0.16 g $Ca(NO_3)_2$, 0.29 g $CaSO_4$, 0.14 g KNO_3, 0.47 g $MgSO_4$, 0.16 g K_2SO_4, 7.5 mg KCl, 3.1 mg H_3BO_3, 0.2 mg H_2MO_4, and 5 mL Fertilome liquid iron). Once poplar stems grew 1-3" roots hydroponically (1 week), roots were coated for 30 min in 60 mL glass tubes with recombinant cultures (overnight 20 mL LB cultures containing 50 μg/mL Km resuspended in 25% sterile modified Hoagland's solution, OD_{600} 5). Poplar stems with coated roots were then planted in 300 mL plastic pots (with drainage holes) which contained 300 g of soil. Pots were watered with 25 mL of

sterile 10% modified Hoagland's solution every 24 hr.

After each week of growth in pots, root samples were obtained by sacrificing a whole plant, cutting 2 cm segments of roots (obtained from the top, middle, and tip root locations), and suspending the root in 3 mL sterile 0.1 M PPB. The 3 mL root suspension was sonicated for 20 sec at 4 watts and then serially diluted with sterile 0.1 M PPB. Dilutions were plated upon LB with or without Km (400 μ g/mL). All the recombinants showed Km resistance up to 400 μg/mL, and all the hosts were sensitive to 50 μg/mL (except *P. fluorescens* 2-79 and S-16H which were sensitive at 100 μg/mL). In addition, 400 μg/mL Km was effective in killing all soil indigenous organisms on LB so plating at this concentration showed only the growth of the recombinants (note that 200 μg/mL Km killed greater than 99.8% of the indigenous bacteria).

5. TOM activity (naphthalene assay)

Recombinant strains grown on roots and engineered to express TOM constitutively were screened weekly for TOM activity based upon the oxidation of naphthalene to naphthol and detection of the purple diazo dye formed after reaction with o-dianisidine (24). The recombinant-containing M9 minimal salts plates with Km were inverted and several naphthalene crystals were added to lid, then the plates were incubated at 30 °C for 30 min. Fresh 0.5% tetrazotized *o*-dianisidine (Sigma Chemical Co.) solution was lightly sprayed upon the cell colonies, after which positive, TOM-expressing colonies turned purple.

6. Trichloroethylene (TCE) initial degradation rates

Recombinant cell cultures (grown overnight in LB containing 50 μg/mL Km) were resuspended in 0.1 M PPB after centrifugation. The cell density was measured and diluted to OD_{600} of 1. Samples were prepared in 60 mL serum vials, each containing 10 mL of the resuspended cells. After capping the vials with Teflon-coated silicone septa (Wheaton, Millville, NJ) and an aluminum crimp seal (Fisher), 10 mM TCE stock solution in *N,N*-dimethylformamide (Fisher) was injected directly into the suspension, using a 10 μL liquid syringe (Hamilton, Reno, NV) to a final concentration of 10 μM TCE (assuming all the TCE dissolved in the liquid phase). The inverted vials were shaken on an IKA KS125 shaker (Munich, Germany) at 300 rpm and at room temperature for 15 min before

taking 50 μL headspace samples at 5 min intervals with a 100 μL gas-tight syringe (Hamilton). The gas samples were injected into a Hewlett Packard (Palo Alto, CA) 5980 Series II gas chromatograph equipped with a flame ionization detector and an Alltech (Deerfield, IL) 0.1% AT-1000 on 80/100 Graphpac packed column (column temperature 200 °C, injector temperature 200 °C, and detector temperature 250 °C; 30 mL/min N_2 used as a carrier gas). To normalize the initial TCE degradation rates, the total cell protein (0.21 - 0.44 mg/mL) was found by using a protein assay kit (Sigma P5656).

7. Stability of TOM expression

The stability of TOM expression of recombinants in suspension cultures was evaluated by measuring the extent of TCE degradation at 10 μM (assuming all the TCE in the liquid phase) after serial dilutions. The recombinants were grown in LB without antibiotic. Serial dilutions were performed every 24 hr by inoculating 20 mL of fresh LB medium with 5 μL of the previous culture, for up to 83 days. Then, a day-old culture was centrifuged at 10,000 rpm for 5 min at 25 °C and cell pellets were resuspended in 0.1 M PPB. After the OD was adjusted to 1 and TCE was injected directly into the 10 mL cell suspension in 60 mL serum vials, the inverted vials were shaken for 24 hr at 300 rpm, then the extent of TCE degradation was determined on a gas chromatograph.

8. Specific growth rates

The maximum specific growth rates of the recombinants and their corresponding hosts were determined using LB without antibiotics. Fifty mL were inoculated with 20 μL of an overnight LB culture, and once the cultures reached an OD_{600} of 0.05, the OD was measured every 15 min until it reached approximately 0.5.

III. RESULTS AND DISCUSSION

To obtain competitive, poplar-colonizing bacteria, unidentified colonies with what appeared to be different morphologies from the poplar rhizosphere were transformed with suicide plasmid pLANT3 and evaluated for TCE degradation, TOM stability, and competitiveness against both the original host as well as

poplar indigenous bacteria. Eleven poplar isolates were obtained from LB plates based on slight color differences and glycocalyx production, and six of these hosts (PM2, PM4, Pb1, Pb2, Pb3, and Pb5) were found to be sensitive to kanamycin at 50 μg/mL, to grow on M9 glucose plates, and to express active TOM once conjugated with pLANT3. The previously-constructed wheat-colonizing recombinant, *P. fluorescens* 2-79TOM, two TCE-degrading constructs from the shrub rhizosphere (S-16 and H-25), and two TCE-degrading *Rhizobium* sp. tree-colonizers were also evaluated for poplar competitiveness.

1. Identification of organisms and specific growth rates

The two most-competitive poplar isolates, Pb3-1 and Pb5-1, were identified using 16S rDNA sequence data (1433 bp and 1446 bp) and BLAST 2.0.11 analysis (1) as *Pseudomonas* species most similar to *Pseudomonas* sp. PsK (GenBank accession number AF105389, 97% and 98% identity); hence, although the hosts appeared slightly different when originally isolated from poplar roots, both transformants are the same strain. *P.* sp. PsK was isolated from the rhizosphere of pinyon junipers which grew in arid woodland sandy-loam soils of Arizona (9). The next nine most similar strains for both of these recombinants were also *Pseudomonas* strains (*P.* sp. 16S, accession number AJ00281; *P. pavonaceae,* D84019; *P. putida* 16, AF095892; *P.* sp., AJ011507; *P. jessenii*, AF068259; *P.* sp. 16S rRNA, X96788; *P. migulae* 1, AF074383; *P. gessardii*, AF074384; *P. libaniens*, AF057645).

The maximum specific growth rates of the TOM-expressing recombinants in suspension were in general found to be similar to those of the respective wild-type organisms indicating little metabolic impact of expressing TOM constitutively (Table 1). Further, recombinants from the same host (e.g., PM2-1, -2, and -3 from PM2 and PM4-1, -2, and 3 from PM4) showed very similar growth rates.

2. Initial degradation rates and stability of TOM expression

As shown in Table 1, the initial TCE degradation rates of the poplar recombinants (PM2-1, PM4-3, Pb1-1, Pb2-1, Pb3-1, and Pb5-1) and the two *Rhizobium* spp. recombinants (10320D and 35645A) were higher than those of *P. fluorescens* 2-79TOM and the two shrub recombinants (S-16 and H-25). These

higher degradation rates are comparable to the previously published values for constitutive expression in the source of TOM, *Burkholderia cepacia* PR123(TOM23C) (26).

The stability of TOM expression and TCE degradation with the recombinants was estimated initially in shake flasks after serial dilution in LB medium (without antibiotic selective pressure) by measuring the extent of overnight TCE degradation. Expression of TOM by these recombinants was very stable and almost 100% of the initial TCE (10 μM) was degraded after 24 hr for a period of up to 83 days (Table 1).

3. Rhizosphere competitiveness of recombinants against hosts

Since seeds were more readily cultivated than poplar trees, the initial competitiveness test (against the host) was performed with both germinated barley and wheat seeds. In these rhizospheres, the poplar recombinants Pb3-1 and Pb5-1, *Rhizobium* sp. recombinant 35645A, and *P. fluorescens* 2-79TOM showed high competitiveness against their corresponding hosts in sterile soil for at least one month (Table 2) since after coating germinated seeds with both the recombinant and host, there was little change in the population in sterile soil over this period for both the barley and wheat rhizospheres. However, one shrub recombinant (S-16) was not competitive against its host in both the barley and wheat rhizospheres, and another shrub recombinant (H-25) showed high competitiveness in the barley rhizosphere but not in wheat rhizosphere (Table 2).

TOM expression in the recombinant strains was maintained for one month for the two poplar isolates Pb3-1 and Pb5-1 since 93 to 100% of the recombinants in the barley and wheat rhizospheres expressed active TOM. TOM activity decreased slightly for *Rhizobium* sp. 35645A in the wheat rhizosphere and significantly for *P. fluorescens* 2-79TOM, S-16, and H-25.

4. Rhizosphere competitiveness of recombinants against indigenous organisms

The poplar rhizosphere competitiveness of 15 recombinants from the poplar, wheat, tree, and scrub rhizospheres was tested by coating poplar tree roots with a single recombinant and planting in non-sterile soil (Table 3). After 4 wks of growth, the percentage of all poplar rhizosphere bacteria which were the

recombinants *P. fluorescens* 2-79TOM (wheat colonizer) or S-16 and H-25 (shrub colonizers) dropped from 100 to less than 0.5%; hence, the recombinants which were not derived from the tree rhizospheres did not thrive there. Furthermore, TOM expression was not maintained by these 3 recombinants (0.8 to 62% of the recombinants actively expressed TOM).

In contrast, all the tree colonizers (excluding poplar recombinant PM4-1), which includes the two *Rhizobium* spp. recombinants (10320D and 35645A) and the poplar-derived recombinants, showed robust poplar rhizosphere competitiveness. Poplar isolates Pb3-1 and Pb5-1 were the most competitive with 79% and 39%, respectively, of the root-associated bacterial population consisting of the recombinant, TCE-degrading strains after four weeks (Table 3). In addition six of these competitive recombinants (including Pb3-1 and Pb5-1) had stable, long-term TOM expression since nearly all the recombinants retained naphthalene oxygenase activity (five strains with 100% of the recombinants manifesting TOM activity). Hence, to create competitive bacteria, it appears beneficial to obtain bacteria from similar rhizospheres or from the actual rhizosphere itself.

These poplar rhizosphere competition results against indigenous bacteria were reproducible since similar results were obtained when four of the original 15 experiments of Table 3 were repeated (with Pb3-1, Pb5-1, *R.* 10320D, and *R.* 35645A). For example, after 28-29 days, in two separate experiments, Pb3-1 was found to have 79 and 63% recombinant cells, respectively, in the rhizosphere with 100% of them expressing active TOM. In addition, recombinant bacteria were found uniformly along the roots since samples taken from three different areas of the poplar root (top, middle, and tip) did not yield differences in cell numbers (only averages for the three regions are shown in Table 3).

These results indicate that a large degradation operon under the control of the IS*50* promoter may be integrated into the chromosome to yield stable, constitutive, and active TOM-expression in a wide-range of bacteria. Further, we have shown that it is possible to create competitive remediation systems by engineering uncharacterized natural strains and then returning these engineered bacteria to the original rhizosphere. The strains developed here should be able to degrade not only TCE but mixtures of TCE and less chlorinated compounds (21), and it is hoped to apply this approach to create a similar system utilizing a related monooxygenase capable of degrading aerobically mixtures including

tetrachloroethylene (15, 20). This approach should be applicable for many enzymes and various pollutants as well as for many Gram-negative bacteria.

Our previous results also showed that rhizoremediation of TCE in soils was accomplished by a rhizosphere established in a defined microcosm by using poplar roots coated with recombinants (unpublished data). In this system, soil excavation expenses are limited to the expenses required for planting, and the need for soil augmentation is minimized since an inducer is unnecessary. The general vectors created in this study may also be used to create other TCE-degrading bacteria capable of colonizing different plant species with perhaps greater root densities, root depths, or water utilization. Therefore, further development of systems incorporating genetically engineered microorganisms (GEMs) promises to yield effective, low-cost rhizoremediation techniques.

참 고 문 헌

1. Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 25:3389-3402.

2. Amann, R.I., W. Ludwig, and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews. 59(1):143-169.

3. Anderson, T.A. and B.T. Walton. 1995. Comparative fate of [^{14}C]trichloroethylene in the root zone of plants from a former solvent disposal site. Environmental Toxicology and Chemistry. 14:2041-2047.

4. Aprill, W. and R.C. Sims. 1990. Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. Chemosphere. 20:253-265.

5. Chappell, J. 1997. Phytoremediation of TCE using *Populus*. http://clu-in.com/phytoTCE.html. U.S. EPA.

6. Crowley, D.E., M.V. Brennerova, C. Irwin, V. Brenner, and D.D. Focht. 1996. Rhizosphere effects on biodegradation of 2,5-dichlorobenzoate by a bioluminescent strain of root-colonizing *Pseudomonas fluorescens*. FEMS Microbiology Ecology. 20:79-89.

7. de Lorenzo, V., S. Fernandez, M. Herrero, U. Jakubzik, and K.N. Timmis. 1993.

Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. Gene. 130:41-46.

8. Fletcher, J.S. and R.S. Hedge. 1995. Release of phenols by perennial plant roots and their potential importance in bioremediation. Environmental Toxicology and Chemistry. 31:3009-3016.

9. Kuske, C., J. Busch, D. Adorada, J. Dunbar, and S. Barns. 1999. Phylogeny, ribosomal RNA gene typing and relative abundance of new *Pseudomonas* species (sensu stricto) isolated from two pinyon-juniper woodland soils of the arid southwest U.S. Systematic and Applied Microbiology. 22(2):300-311.

10. Luu, P.P., C.W. Yung, A.K-Y. Sun, and T.K. Wood. 1995. Monitoring trichloroethylene mineralization by *Pseudomonas cepacia* G4 PR1. Applied Microbiology and Biotechnology. 44:259-264.

11. McCarty, P.L. 1997. Breathing with chlorinated solvents. Science. 276:1521-1522.

12. Nelson, M.J.K., S.O. Montgomery, E.J. O'Neill, and P.H. Pritchard. 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. Applied and Environmental Microbiology. 52(2):383-384.

13. Newman, L.M. and L.P. Wackett. 1995. Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry. 34:14066-14076.

14. Radwan, S., N. Sorkhoh, and I. El-Nemr. 1995. Oil biodegradation around roots. Nature. 376:302.

15. Ryoo, D., H. Shim, K. Canada, P. Barbieri, and T.K. Wood. 2000. Aerobic degradation of tetrachloroethylene by toluene-*o*-xylene-monooxygenase of *Pseudomomas stutzeri* OX1. Nature Biotechnology. 18(7):775-778.

16. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning, A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

17. Shields, M.S. and S.C. Francesconi. 6 August, 1996. U.S. Patent 5,543,317.

18. Shields, M.S. and S.C. Francesconi. 1996. Molecular techniques in bioremediation, pp.341-390. *In* R.L. Crawford and D.L. Crawford (ed.), Bioremediation: Principles and applications. Cambridge University Press, Cambridge.

19. Shields, M.S., M.J. Reagin, R.R. Gerger, C. Somerville, R. Schaubhut, R.

Campbell, and J. Hu-Primmer. 1994. Constitutive degradation of trichloroethylene by an altered bacterium in a gas-phase bioreactor, pp.50-65. *In* R.E. Hinchee, A. Leeson, L. Semprini, and S.K. Ong (ed.), Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds. Lewis Publishers, Boca Raton.

20. Shim, H., D. Ryoo, P. Barbieri, and T.K. Wood. 2000. Aerobic degradation of mixtures of tetrachloroethylene, trichloroethylene, dichloroethylenes, and vinyl chloride by toluene-*o*-xylene-monooxygenase of *Pseudomonas stutzeri* OX1. Applied Microbiology and Biotechnology. In review.

21. Shim, H. and T.K. Wood. 2000. Aerobic degradation of mixtures of chlorinated aliphatics by cloned toluene-*o*-xylene-monooxygenase and toluene *o*-monooxygenase in resting cells. Biotechnology and Bioengineering. In press (December, 2000).

22. Shurtliff, M.M., G.F. Parkin, L.J. Weathers, and D.T. Gibson. 1996. Biotransformation of trichloroethylene by a phenol-induced mixed culture. ASCE Journal of Environmental Engineering. 122:581-589.

23. Stomp, A.M., K.H. Han, S. Wilbert, and M.P. Gordon. 1993. Genetic improvement of tree species for remediation of hazardous wastes. In Vitro Cellular and Developmental Biology. 29:227-232.

24. Wackett, L.P. and D.T. Gibson. 1983. Rapid method for detection and quantification of hydroxylated aromatic intermediates produced by microorganisms. Applied and Environmental Microbiology. 45(3):1144-1147.

25. Walton, B.T. and T.A. Anderson. 1990. Microbial degradation of trichloroethylene in the rhizosphere: Potential application to biological remediation of waste sites. Applied and Environmental Microbiology. 56(4):1012-1016.

26. Yee, D.C., J.A. Maynard, and T.K. Wood. 1998. Rhizoremediation of trichloroethylene by a recombinant, root-colonizing *Pseudomonas fluorescens* strain expressing toluene *ortho*-monooxygenase constitutively. Applied and Environmental Microbiology. 64(1):112-118.