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Rhizosphere Competitiveness of Trichloroethylene-Degrading, Poplar-Colonizing Recombinant Bacteria

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Indigenous bacteria from poplar tree (*Populus canadensis* var. *eugenei* 'Imperial Carolina') and southern California shrub rhizospheres, as well as two tree-colonizing *Rhizobium* strains (ATCC 10320 and ATCC 35645), were 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* recombinant bacteria degraded trichloroethylene at a rate of 0.8 to 2.1 nmol/min/mg of protein and were competitive against the unengineered hosts in wheat and barley rhizospheres for 1 month (colonization occurred at a level of 1.0 × 10^5 to 23 × 10^5 CFU/cm of root). In addition, six of these recombinants colonized poplar roots stably and competitively with populations as large as 79% ± 12% of all rhizosphere bacteria after 28 days (0.2 × 10^5 to 31 × 10^5 CFU/cm of root). Furthermore, five of the most competitive poplar recombinants (e.g., Pb3-1 and Pb5-1, which were identified as *Pseudomonas* sp. strain PsK recombinants) retained the ability to express TOM for 29 days as 100% ± 0% of the recombinants detected in the poplar rhizosphere expressed TOM constitutively.

Plants are useful for bioremediation. It has been known for at least 70 years that some species accumulate toxic metals (21), and some plants have been shown to stimulate degradation of polycyclic aromatic hydrocarbons (4) and 2,5-dichlorobenzoate (5). In Kuwait, many crop species have been shown to grow in soil containing up to 10% crude oil by weight and to cleanse the rhizosphere of the crude oil (13). For chlorinated aliphatic compounds, 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). TCE is a suspected carcinogen and is one of the most common groundwater pollutants at hazardous waste sites (10).

To take advantage of the plant-bacterium relationship to degrade chlorinated aliphatic compounds, the genus *Populus* (which includes poplars, cottonwoods, and aspens [http://clu-in.com/phytoTCE.htm]) was chosen for this work since these trees have many advantages for rhizoremediation. Poplars grow quite rapidly (3 to 5 m/year) because 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 (http://clu-in.com/phytoTCE.htm). A 5-year-old tree can process over 53 gal of water per day (http://clu-in.com/phytoTCE.htm); hence, each day 53 gal 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 (17). However, the rhizosphere provides controlled conditions for symbiotic growth of 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, 23); hence, engineered rhizobacteria have a niche in which to flourish (the bacterial populations in the rhizosphere are 2 to 3 orders of magnitude larger than those in the outlying soil [4]). Since high levels of phenolic compounds have been found in root exudates (7), these compounds also induce bacterial dioxygenase remediation pathways (20). Along with providing the bacteria with both nutrients and oxygen for chlorinated aliphatic compound degradation, the trees also transport the bacteria throughout 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*) (16) 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 (12). TOM oxidizes TCE, all three dichloroethylenes, and vinyl chloride (16, 18), and TCE is degraded primarily to CO_2 and Cl^- in vivo (9, 11). Recent studies also indicate that strains expressing TOM can degrade mixtures of these compounds (19).

To create a general cloning vector capable integrating the genes for the TCE-degrading TOM enzyme into virtually any gram-negative bacterial chromosome, the 11.3-kb DNA fragment containing kanamycin resistance and the constitutive IS*50* promoter of Tn*5* (16) fused to *tomA012345* was ligated into the suicide Tn*5* transposon plasmid pCNB4 (6) to form pLANT3 (24). pLANT3 does not replicate in non-λ*pir* lyso-

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TABLE 1. Growth rates, initial TCE degradation rates, and shake flask stability of TCE-degrading recombinants

Recombinant[a]	Source of host	Specific growth rate (h^{-1})		Initial TCE degradation rate (nmol/min/mg of protein)	Minimum stability (days)[b]
		Recombinant	Host		
PM2-1	Poplar root from Michigan	0.967	0.911	1.68	7
PM2-2	Poplar root from Michigan	0.956			7
PM2-3	Poplar root from Michigan	0.986			7
PM4-1	Poplar root from Michigan	0.720	0.723		7
PM4-2	Poplar root from Michigan	0.730			7
PM4-3	Poplar root from Michigan	0.737		1.37	7
Pb1-1	Poplar root from Michigan	0.808	0.853	1.51	7
Pb2-1	Poplar root from Michigan	0.867	1.024	2.09	7
Pb3-1	Poplar root from Michigan	0.949	1.179	1.15	7
Pb5-1	Poplar root from Michigan	1.056	1.006	0.83	7
Rhizobium sp.					
10320D	ATCC 10320	0.727	0.792	1.90	7
35645A	ATCC 35645	0.805	0.593	1.21	7
P. fluorescens 2-79TOM	*P. fluorescens* 2-79	0.754	0.770	0.50	36
S-16	Shrub root from California	0.815	ND[c]	0.21	77
H-25	Shrub root from California	1.066	ND	0.35	83

[a] PM2, PM4, Pb1, Pb2, Pb3, and Pb5 are hosts obtained from poplar roots, and the last numbers of the recombinant designations indicate different transformants of these hosts which actively expressed TOM. All recombinants were resistant to kanamycin at concentrations up to 400 μg/ml. All poplar hosts and the hosts for H-25 and the two *Rhizobium* spp. recombinants were sensitive to 50 μg of kanamycin per ml, whereas *P. fluorescens* 2-79 and the host for S-16 were resistant to 50 μg of kanamycin per ml but sensitive to 100 μg of kanamycin per ml.

[b] All recombinants showed 100% TCE (10 μM) degradability after 24 h with resting cells (after serial dilution in LB medium without an antibiotic).

[c] ND, not determined.

gens; after conjugation, a single transposition event occurs to integrate the *tom* locus and kanamycin resistance into the chromosome. This method was used previously to form the wheat root-colonizing strain *Pseudomonas fluorescens* 2-79TOM (24), which continuously expressed active TOM at high levels (the enzyme was stable for up to 290 generations) and which degraded TCE in wheat rhizospheres (24).

In this study, in order 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 the ability to compete in the poplar tree rhizosphere and stably express TOM. This is the first report of construction of tree-colonizing bacteria for bioremediation.

MATERIALS AND METHODS

Microorganisms. Uncharacterized poplar tree-colonizing bacteria (Table 1, PM and Pb strains) were obtained by sonicating roots of hybrid poplar (*Populus canadensis* var. *eugenei* 'Imperial Carolina' from Vans Pines Nursery, West Olive, Mich., and Segal Ranch, Grandview, Wash.) for 20 s at 4 W with a Sonic Dismembrator (model 60; Fisher Scientific Company, Fair Lawn, N.J.), plating the resulting solution on Luria-Bertani (LB) medium (15), and incubating the plates at 30°C. The roots of two native shrubs from Crystal Cove, Calif., were similarly treated, and two isolates (isolates S and H) were obtained. The wheat colonizers *P. fluorescens* 2-79 and 2-79TOM (24), which express TOM constitutively, were used along with two tree colonizers obtained from the American Type Culture Collection (Rockville, Md.): *Rhizobium* strains ATCC 10320 (from black locust [*Robina pseudoacacia*]) and ATCC 35645 (from Haole koa [*Leucaena leucocephala*]).

Phylogenetic identification of organisms. The two most competitive poplar recombinants, Pb3-1 and Pb5-1, were identified by 16S ribosomal DNA (rDNA) analysis. Bacterial genomic DNA was extracted by a Triton-prep method. In this procedure 5 ml of an overnight culture was mixed with 300 μl of STET (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl [pH 8.0], 50 mM EDTA [pH 8.0]), 20 μl of a solution containing 50 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml, and 10 μl of a solution containing 10 mg of RNase A (Sigma Chemical Co.) per ml. This mixture was boiled for 1.5 min and centrifuged for 15 min, and the supernatant was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) (Fisher Scientific). The DNA in the aqueous phase was precipitated with isopropanol and washed with 70% ethanol. After vacuum drying, the DNA was suspended in 200 μl of water. PCR were performed with 5-μl portions of the genomic DNA in 50-μl reaction mixtures by using standard cycling conditions (94°C for 5 min and 30 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 10 min at 72°C). The following two sets of universal 16S rDNA primers were synthesized by Gibco BRL (Gaithersburg, Md.) (positions on the *Escherichia coli* chromosome are indicated): HK12 (2) (5′-GAGTTTGATCCTGGCTCAG; positions 10 to 28) and HK13 (5′-TACCTTGTTACGACTT; positions 1492 to 1507); and JCR15 (2) (GCCA GCAGCCGCGGTA; positions 517 to 532) and JCR14 (2) (ACGGGCGGTGT GTAC; positions 1392 to 1406). Primers HK12 and HK13 amplified the entire 16S rDNA region (1,497 bp), while primers JCR14 and JCR15 amplified 889 bp within the 16S rDNA. Each set of primers was used in triplicate PCR for each isolate so that there was twofold redundancy to avoid errors (two 16S rDNA regions, and each region was sequenced three times). The PCR products were purified with a Qia-Quick PCR purification kit, and the DNA was sequenced with an automated ABI automated sequencer.

Conjugation. Based on colony morphology, eight different hosts obtained from the LB medium plates containing the isolates from the poplar and native shrub roots which showed growth on glucose M9 minimal salts (15) but no growth on LB medium containing 50 μg of kanamycin per ml were conjugated with the donor *E. coli* S17-1(λ*pir*)/pLANT3 containing *tomA012345* as described previously (24) to create PM2-1, PM2-2, PM2-3, PM4-1, PM4-2, PM4-3, Pb1-1, Pb2-1, Pb3-1, Pb5-1, S-16, and H-25 (Table 1). Two *Rhizobium* recombinants expressing TOM (10320D and 35645A) were obtained similarly after their corresponding hosts (*Rhizobium* strains ATCC 10320 and ATCC 35645) were conjugated with *E. coli* S17-1(λ*pir*)/pLANT3. Only one transposition event occurs (6) with pLANT3 since the enzyme for transposition does not integrate along with *tomA012345*.

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 the corresponding wild-type hosts was tested in barley and wheat rhizospheres in sterile soil. Seeds of Moongold barley (Liberty Seed Company, New Philadelphia, Ohio) and Cavalier winter wheat (Stover Seed Co., Los Angeles, Calif.) were sterilized first with 95% ethanol for 10 s and then with 3% hydrogen peroxide for 3 min and then were germinated by incubation on wet sponges for 2 to 5 days at room temperature in the dark. The sponges were sterilized by soaking them in a 2.5% sodium hypochloride solution for 30 min, rinsed with distilled water, and then individually autoclaved in a foil-covered beaker containing water (the sponges were 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 medium to

a final optical density at 600 nm (OD_{600}) of 1.4 to 1.6 (recombinant with 50 μg of kanamycin per ml). After centrifugation at 13,800 × *g* for 5 min at 25°C, both cell pellets were resuspended in 0.1 M potassium phosphate buffer (PPB) (pH 7); then the OD_{600} was adjusted to the same value for both the recombinant and the 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 h. Ten to 15 seeds were planted with 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, Ohio), illuminated for 16 h each day, and watered with 25 ml of sterile tap water every 48 h.

The competitiveness of 15 of the recombinants against indigenous organisms present in nonsterile soil (from Mansfield Center, Conn.) was determined in the poplar rhizosphere. Poplar stems that were 6 in. long were cut and grown hydroponically in order to obtain roots in 25% modified Hoagland's solution, which contained (per liter) 0.21 g of KH_2PO_4, 0.16 g of $Ca(NO_3)_2$, 0.29 g of $CaSO_4$, 0.14 g of KNO_3, 0.47 g of $MgSO_4$, 0.16 g of K_2SO_4, 7.5 mg of KCl, 3.1 mg of H_3BO_3, 0.2 mg of H_2MO_4, and 5 ml of Fertilome liquid iron. Once the poplar stems grew 1- to 3-in.-long roots hydroponically (1 week), the roots were coated for 30 min in 60-ml glass tubes with recombinant cultures (overnight 20-ml LB medium cultures containing 50 μg of kanamycin per ml resuspended in 25% sterile modified Hoagland's solution; OD_{600} of 5). Poplar stems with coated roots were then planted in 300-ml plastic pots (with drainage holes) which contained 300 g of soil. The pots were watered with 25 ml of sterile 10% modified Hoagland's solution every 24 h.

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), and suspending the root segments in 3 ml of sterile 0.1 M PPB. Each 3-ml root suspension was sonicated for 20 s at 4 W and then serially diluted with sterile 0.1 M PPB. Dilutions were plated on LB medium with or without kanamycin (400 μg/ml). All of the recombinants showed resistance to kanamycin at concentrations up to 400 μg/ml, and most of the hosts were sensitive to 50 μg of kanamycin per ml (the exceptions were *P. fluorescens* 2-79 and S-16H, which were sensitive to 100 μg/ml). In addition, 400 μg of kanamycin per ml effectively killed all indigenous soil organisms on LB medium so plating with this concentration resulted in growth of only the recombinants (note that 200 μg of kanamycin per ml killed more than 99.8% of the indigenous bacteria).

TOM activity (naphthalene assay). Recombinant strains grown on roots and engineered to express TOM constitutively were screened weekly for TOM activity based on the oxidation of naphthalene to naphthol and detection of the purple diazo dye formed after reaction with *o*-dianisidine (22). Recombinant-containing M9 minimal salts plates with kanamycin were inverted, and several naphthalene crystals were added to each lid; then the plates were incubated at 30°C for 30 min. A fresh 0.5% tetrazotized *o*-dianisidine (Sigma Chemical Co.) solution was lightly sprayed on the cell colonies, after which positive, TOM-expressing colonies turned purple.

Initial TCE degradation rates. Recombinant cell cultures (grown overnight in LB medium containing 50 μg of kanamycin per ml) were resuspended in 0.1 M PPB after centrifugation. The cell densities were measured, and each culture was diluted to an OD_{600} of 1. Samples were prepared in 60-ml serum vials, each of which contained 10 ml of the resuspended cells. After the vials were capped with Teflon-coated silicone septa (Wheaton, Millville, N.J.) and aluminum crimp seals (Fisher Scientific), a 10 mM TCE stock solution in *N,N*-dimethylformamide (Fisher Scientific) was injected directly into the suspension with a 10-μl liquid syringe (Hamilton, Reno, Nev.) to a final TCE concentration of 10 μM (assuming that all the TCE dissolved in the liquid phase). The inverted vials were shaken with a KS125 shaker (IKA, Munich, Germany) at 300 rpm at room temperature for 15 min before 50-μl headspace samples were removed at 5-min intervals with a 100-μl gas-tight syringe (Hamilton). The gas samples were injected into a Hewlett-Packard (Palo Alto, Calif.) 5980 series II gas chromatograph equipped with a flame ionization detector and an Alltech (Deerfield, Ill.) 0.1% AT-1000 on 80/100 Graphpac packed column (column temperature, 200°C; injector temperature, 200°C; detector temperature, 250°C; 30 ml/min; N_2 used as the carrier gas). To normalize the initial TCE degradation rates, the total cell protein concentration (0.21 to 0.44 mg/ml) was determined with a protein assay kit (catalog no. P5656; Sigma Chemical Co.).

Stability of TOM expression. The stability of TOM expression by recombinants in suspension cultures was evaluated by measuring the extent of degradation of 10 μM TCE (assuming that all the TCE was in the liquid phase) after serial dilution. The recombinants were grown in LB medium without an antibiotic. Serial dilutions were prepared every 24 h by inoculating 20 ml of fresh LB medium with 5 μl of the previous culture for up to 83 days. Then, 1-day-old cultures were centrifuged at 13,800 × *g* for 5 min at 25°C, and the cell pellets were resuspended in 0.1 M PPB. After the OD_{600} were adjusted to 1 and TCE was injected directly into 10-ml cell suspensions in 60-ml serum vials, the vials were inverted and shaken for 24 h at 300 rpm; then the extent of TCE degradation was determined with a gas chromatograph.

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

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 and bacteria indigenous to the poplar rhizosphere. Eleven poplar isolates were obtained from LB medium 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 they were conjugated with pLANT3. The previously constructed wheat-colonizing recombinant, *P. fluorescens* 2-79TOM (24), along with 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.

Identification of organisms and specific growth rates. The two most competitive poplar isolates, Pb3-1 and Pb5-1, were identified by using 16S rDNA sequence data (1,433 and 1,446 bp) and BLAST 2.0.11 analysis (1) as members of the genus *Pseudomonas* most similar to *Pseudomonas* sp. strain PsK (GenBank accession number AF105389) (97 and 98% identity); hence, although the hosts appeared slightly different when they were originally isolated from poplar roots, these two transformants are the same strain. *Pseudomonas* sp. strain PsK was isolated from the rhizosphere of pinyon junipers which grew in arid woodland soils of Arizona (8). The nine strains most similar to both of these recombinants were also *Pseudomonas* strains (*Pseudomonas* sp. strain 16S [accession number AJ00281], *P. pavonaceae* [D84019], *P. putida* 16 [AF095892], *Pseudomonas* sp. [AJ011507], *P. jessenii* [AF068259] *Pseudomonas* sp. 16S rRNA [X96788], *P. migulae* 1 [AF074383], *P. gessardii* [AF074384], and *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 that there was little metabolic impact of constitutive TOM expression (Table 1). Furthermore, recombinants from the same host (e.g., PM2-1, PM2-2, and PM2-3 from PM2 and PM4-1, PM4-2, and PM4-3 from PM4) showed very similar growth rates.

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, *B. cepacia* $PR1_{23}(TOM_{23C})$ (24).

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 recombinants was very stable, and almost 100% of the initial TCE (10 μM) was degraded after 24 h for up to 83 days (Table 1).

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 germinated barley and wheat seeds. In the rhizospheres of these plants, poplar recombinants Pb3-1 and Pb5-1, *Rhizobium* sp. recombinant 35645A, and *P. fluorescens* 2-79TOM showed high levels of competitiveness against their corresponding

TABLE 2. Competitiveness of two poplar recombinants (Pb3-1 and Pb5-1), a *Rhizobium* recombinant (35645A), *P. fluorescens* 2-79TOM, and two shrub recombinants (S-16 and H-25) against their respective hosts in rhizospheres of barley and wheat in sterile soil

Recombinant	Plant	Days after planting	% of recombinants	% of active TOM in recombinants	Total no. of organisms (CFU/cm of root or CFU/ml of PPB)[a]
Pb3-1	Barley	0	58	100	2.4×10^{10}
		7	56 ± 40[b]	86 ± 17	$8.0 \times 10^5 \pm 6.5 \times 10^5$
		14	49 ± 10	74 ± 18	$8.5 \times 10^5 \pm 7.3 \times 10^5$
		21	57 ± 7	100 ± 0	$1.7 \times 10^5 \pm 1.7 \times 10^5$
		28	68 ± 27	100 ± 0	$1.8 \times 10^5 \pm 2.5 \times 10^5$
	Wheat	0	55	100	10.4×10^{10}
		7	90 ± 8	100 ± 0	$6.2 \times 10^5 \pm 9.4 \times 10^5$
		14	71 ± 27	99 ± 2	$23 \times 10^5 \pm 10 \times 10^5$
		21	57 ± 23	96 ± 6	$2.2 \times 10^5 \pm 1 \times 10^5$
		28	48 ± 9	93 ± 12	$1.3 \times 10^5 \pm 1.5 \times 10^5$
Pb5-1	Barley	0	47	100	6.8×10^{10}
		7	75 ± 5	100 ± 0	$14 \times 10^5 \pm 9 \times 10^5$
		14	47 ± 24	96 ± 4	$30 \times 10^5 \pm 12 \times 10^5$
	Wheat	0	47	100	1.7×10^{10}
		7	46 ± 17	100 ± 0	$25 \times 10^5 \pm 19 \times 10^5$
		15	73 ± 36	100 ± 0	$10 \times 10^5 \pm 5 \times 10^5$
		21	80 ± 21	100 ± 0	$7.2 \times 10^5 \pm 6.8 \times 10^5$
		28	60 ± 10	100 ± 0	$0.98 \times 10^5 \pm 0.59 \times 10^5$
Rhizobium sp. strain 35645A	Barley	0	30	100	29.7×10^{10}
		7	54 ± 18	39 ± 10	$8.2 \times 10^5 \pm 7 \times 10^5$
		14	80 ± 10	100 ± 0	$31 \times 10^5 \pm 11 \times 10^5$
		28	51 ± 31	91 ± 10	$3.5 \times 10^5 \pm 3.7 \times 10^5$
	Wheat	0	63	100	9.0×10^{10}
		7	24 ± 2	96 ± 7	$53 \times 10^5 \pm 26 \times 10^5$
		15	53 ± 28	93 ± 12	$33 \times 10^5 \pm 38 \times 10^5$
		21	60 ± 24	100 ± 0	$12 \times 10^5 \pm 2.7 \times 10^5$
		28	43 ± 20	82 ± 10	$5.9 \times 10^5 \pm 5.4 \times 10^5$
2-79TOM[c]	Barley	0	28	100	2.7×10^{10}
		10	33	33	100×10^5
		25	31	80	4×10^5
	Wheat	0	28	100	2.7×10^{10}
		10	22	33	50×10^5
		17	12	63	2.2×10^5
		25	16	33	9.5×10^5
S-16[c]	Barley	0	55	100	1.9×10^9
		10	22	67	50×10^5
		17	5	23	77×10^5
		25	16	57	22×10^5
	Wheat	0	55	100	1.9×10^9
		10	20	50	400×10^5
		17	9	75	17×10^5
		25	5	67	6×10^5
H-25[c]	Barley	0	93	100	5.7×10^{10}
		10	75	75	100×10^5
		17	93	27	52×10^5
		25	93	86	23×10^5
	Wheat	0	93	100	5.7×10^{10}
		10	35	75	140×10^5
		17	33	100	1.5×10^5
		25	31	25	6.5×10^5

[a] The zero-time data are expressed in CFU per milliliter of PPB; all other data are expressed in CFU per centimeter of root.
[b] Mean ± standard deviation.
[c] Only one 2-cm root sample was analyzed for each plant for these strains on each sampling day.

TABLE 3. Competitiveness of TCE-degrading recombinants against indigenous soil organisms in the poplar rhizosphere[a]

Recombinant	Days after planting	% of recombinants	% of active TOM	Total no. of organisms, (10^5 CFU/cm of root)
Pb3-1	7	30 ± 10[b]	95 ± 8	11 ± 8
	14	47 ± 37	46 ± 45	0.3 ± 0.3
	21	48 ± 20	62 ± 12	2 ± 2
	28	79 ± 12	100 ± 0	0.2 ± 0.1
	7	30 ± 13	99 ± 0	10 ± 8
	14	41 ± 15	97 ± 5	3.4 ± 2
	29	63 ± 37	100 ± 0	4.2 ± 2
Pb2-1	7	46 ± 5	100 ± 0	7 ± 9
	14	18 ± 8	91 ± 14	15 ± 13
	29	39 ± 3	100 ± 0	0.5 ± 0.5
Pb5-1	7	35 ± 2	100 ± 0	5 ± 4
	14	5 ± 6	25 ± 21	29 ± 44
	29	39 ± 20	100 ± 0	0.9 ± 0.8
	7	28 ± 24	100 ± 0	5 ± 5
	15	21 ± 12	89 ± 19	0.9 ± 0.7
PM2-1	9	26 ± 10	100 ± 0	46 ± 5
	14	31 ± 21	89 ± 19	2 ± 1
	22	12 ± 8	93 ± 12	5 ± 1
	29	25 ± 22	65 ± 22	4 ± 4
PM2-2	9	21 ± 5	93 ± 5	39 ± 3
	14	29 ± 22	77 ± 40	3 ± 3
	22	5 ± 4	74 ± 25	0.9 ± 0.7
	29	25 ± 21	100 ± 0	4 ± 6
Pb1-1	7	62 ± 17	100 ± 0	13 ± 13
	14	48 ± 32	100 ± 0	3 ± 3
	29	19 ± 24	67 ± 58	0.6 ± 0.8
PM2-3	9	13 ± 6	100 ± 0	83 ± 26
	14	17 ± 20	89 ± 19	16 ± 12
	22	36 ± 21	100 ± 0	7 ± 4
	29	7 ± 6	87 ± 12	2 ± 1
PM4-3	9	25 ± 5	100 ± 0	35 ± 27
	14	34 ± 18	65 ± 18	22 ± 14
	22	25 ± 4	7 ± 3	10 ± 12
	29	15 ± 4	52 ± 60	7 ± 3
PM4-2	9	21 ± 5	100 ± 0	53 ± 4
	14	1 ± 1	79 ± 26	34 ± 43
	22	4 ± 3	67 ± 56	3 ± 3
	29	13 ± 21	33 ± 60	4 ± 5
PM4-1	9	25 ± 3	96 ± 2	34 ± 25
	14	7 ± 9	86 ± 20	2 ± 2
	22	16 ± 6	41 ± 52	1 ± 1
	29	0.9 ± 1.0	38 ± 54	2 ± 1
Rhizobium sp. strain 10320D	8	25 ± 31	99 ± 1	4 ± 5
	14	9 ± 6	100 ± 0	118 ± 62
	22	34 ± 20	92 ± 13	2 ± 2
	28	7 ± 8	48 ± 47	2 ± 2
	7	29 ± 9	100 ± 0	1.8 ± 1
	14	62 ± 46	99 ± 1	39 ± 42
Rhizobium sp. strain 35645A	7	68 ± 11	100 ± 0	2 ± 1
	14	25 ± 14	100 ± 0	5 ± 8
	21	36 ± 10	100 ± 0	1 ± 0.5
	25	37 ± 17	100 ± 0	1 ± 1
	7	46 ± 40	99.6 ± 0	3 ± 2
	14	72 ± 10	98 ± 2	105 ± 20
	21	19 ± 22	98 ± 2	8 ± 6
	28	17 ± 28	100 ± 0	3 ± 1
P. fluorescens 2-79TOM	7	3 ± 4	50 ± 41	2 ± 1
	14	1 ± 0.6	7 ± 9	5 ± 7
	21	2 ± 3	30 ± 52	59 ± 26
	28	0.1 ± 0.1	22 ± 38	12 ± 6
S-16	7	18 ± 10	92 ± 5	2 ± 1
	14	11 ± 6	92 ± 5	3 ± 1
	21	0.7 ± 0.4	32 ± 32	2 ± 1
	28	0.4 ± 0.2	62 ± 33	31 ± 23
H-25	7	43 ± 30	13 ± 5	6 ± 1
	14	0.3 ± 0.2	15 ± 15	6 ± 6
	21	1 ± 0.9	57 ± 33	25 ± 22
	28	0.3 ± 0.4	0.8 ± 1	14 ± 7

[a] The results of two independent experiments are shown for some strains.
[b] Mean ± standard deviation.

hosts in sterile soil for at least 1 month (Table 2) since after germinated seeds were coated 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 a high level of competitiveness in the barley rhizosphere but not in the wheat rhizosphere (Table 2).

TOM expression in the recombinant strains was maintained for 1 month for 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. strain 35645A in the wheat rhizosphere and significantly for *P. fluorescens* 2-79TOM, S-16, and H-25.

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 single recombinants and planting them in nonsterile soil (Table 3). After 4 weeks of growth, the percentage of all poplar rhizosphere bacteria which were the recombinant *P. fluorescens* 2-79TOM (wheat colonizer) or the recombinants S-16 and H-25 (shrub colonizers) decreased 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 three recombinants (0.8 to 62% of the recombinants actively expressed TOM).

In contrast, all of the tree colonizers (except poplar recombinant PM4-1), including 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; 79 and 39%, respectively, of the root-associated bacterial population consisted of the recombinant, TCE-degrading strains after 4 weeks (Table 3). In addition, six of the competitive recombinants

(including Pb3-1 and Pb5-1) had stable, long-term TOM expression since nearly all of the recombinants retained naphthalene oxygenase activity (five strains, with 100% of the recombinants manifesting TOM activity). Hence, to create competitive bacteria, it appears to be beneficial to obtain bacteria from similar rhizospheres or from the actual rhizosphere itself.

These results of poplar rhizosphere competition against indigenous bacteria were reproducible since similar results were obtained when 4 of the original 15 experiments shown in Table 3 were repeated (with Pb3-1, Pb5-1, and *Rhizobium* spp. strains 10320D and 35645A). For example, after 28 to 29 days, in two separate experiments, Pb3-1 was found to account for 79 and 63% of the recombinant cells in the rhizosphere, with 100% of the cells expressing active TOM. In addition, recombinant bacteria were found uniformly along the roots; samples taken from three different areas of the poplar roots (top, middle, and tip) did not yield different cell numbers (only averages for the three regions are shown in Table 3).

Our results indicate that a large degradation operon under control of the IS*50* promoter may be integrated into the chromosome to obtain stable, constitutive, active TOM expression in a wide range of bacteria. Furthermore, we found that it is possible to create competitive rhizoremediation systems by engineering uncharacterized natural strains and then returning the engineered bacteria to the original rhizosphere. The strains developed here should be able to degrade not only TCE but also mixtures of TCE and less chlorinated compounds (19), and it is hoped that this approach can be used to create a similar system with a related monooxygenase capable of aerobically degrading mixtures that include tetrachloroethylene (14; H. Shim, D. Ryoo, P. Barbieri, and T. K. Wood, submitted for publication). This approach should be applicable to many enzymes and various pollutants, as well as to many gram-negative bacteria.

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