APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1998, p. 112–118 Vol. 64, No. 1
0099-2240/98/$04.00+0

Rhizoremediation of Trichloroethylene by a Recombinant, Root-Colonizing *Pseudomonas fluorescens* Strain Expressing Toluene *ortho*-Monooxygenase Constitutively

DENNIS C. YEE, JENNIFER A. MAYNARD,† AND THOMAS K. WOOD*

Department of Chemical and Biochemical Engineering, University of California, Irvine, Irvine, California 92697-2575

Received 11 July 1997/Accepted 2 October 1997

Trichloroethylene (TCE) was removed from soils by using a wheat rhizosphere established by coating seeds with a recombinant, TCE-degrading *Pseudomonas fluorescens* strain that expresses the *tomA*$^+$ (toluene *o*-monooxygenase) genes from *Burkholderia cepacia* PR1$_{23}$(TOM$_{23C}$). A transposon integration vector was used to insert *tomA*$^+$ into the chromosome of *P. fluorescens* 2-79, producing a stable strain that expressed constitutively the monooxygenase at a level of 1.1 nmol/min · mg of protein (initial TCE concentration, 10 μM, assuming that all of the TCE was in the liquid) for more than 280 cell generations (36 days). We also constructed a salicylate-inducible *P. fluorescens* strain that degraded TCE at an initial rate of 2.6 nmol/min · mg of protein in the presence of 10 μM TCE [cf. *B. cepacia* G4 PR1$_{23}$(TOM$_{23C}$), which degraded TCE at an initial rate of 2.5 nmol/min · mg of protein]. A constitutive strain, *P. fluorescens* 2-79TOM, grew (maximum specific growth rate, 0.78 h^{-1}) and colonized wheat (3 × 10^6 CFU/cm of root) as well as wild-type *P. fluorescens* 2-79 (maximum specific growth rate, 0.77 h^{-1}; level of colonization, 4 × 10^6 CFU/cm of root). Rhizoremediation of TCE was demonstrated by using microcosms containing the constitutive monooxygenase-expressing microorganism, soil, and wheat. These closed microcosms degraded an average of 63% of the initial TCE in 4 days (20.6 nmol of TCE/day · plant), compared to the 9% of the initial TCE removed by negative controls consisting of microcosms containing wild-type *P. fluorescens* 2-79-inoculated wheat, uninoculated wheat, or sterile soil.

The term rhizosphere refers to the environment influenced by plant roots in which elevated bacterial activity is observed (13). 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 microorganism to pollutants 10 to 15 m deep in the soil (27, 45). The roots supply nutrients (amino acids, carbohydrates, and organic acids) (1, 3, 29) so no exogenous carbon source must be added, and they may also supply bacteria with cofactors required for the activation of bacterial enzymes involved in the pollutant degradation pathway. Root-colonizing bacteria genetically engineered to degrade pollutants should also 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 (6); 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. A consortium isolated from the rhizosphere of wheat utilized the herbicide mecoprop as a sole carbon and energy source (28), and bacteria from rhizosphere soils amended with succinic acid and formic acid produced 5 to 10 times more $^{14}CO_2$ from mineralization of radiolabelled pyrene than nonsterile control soils produced (44). Soils planted with rice degraded four times more parathion to CO_2 than unplanted soils degraded (41). Up to three times more trichloroethylene (TCE) was mineralized to CO_2 in rhizosphere soil and plant samples from TCE-contaminated waste sites than in samples from nonvegetated soils (2).

Although employing indigenous microorganisms and plants to treat contaminated soils often requires the identification of suitable, preexisting natural systems (58), bacteria known to colonize specific plants may be engineered to gain more control over the process. A genetically stable, root-colonizing *Pseudomonas fluorescens* strain, for example, was constructed by chromosomal integration of the *bph* genes (encoding polychlorinated biphenyl degradation) with a suicide integration vector (6). Degradation of polychlorinated biphenyls with this recombinant bacterium was demonstrated in shake flask studies, and in situ expression was observed in bacterium-plant-soil microcosms by using a reporter gene fused to the inserted biphenyl-degrading genes (6). A different strain of *P. fluorescens* was also created, and soil mixed with this recombinant bacterium and planted with beans degraded 2,5-dichlorobenzoate by using it as a carbon and energy source (12).

Since World War II, TCE has been used as the standard solvent for degreasing metal parts and textiles (16, 39, 56), and demand for TCE may increase with U.S. Environmental Protection Agency approval of TCE as an alternative for chlorofluorocarbon 113 and methyl chloroform (20). TCE is now present in 27.9% of hazardous waste sites (it is one of the 10 most common pollutants detected at such sites) (8), 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 (8, 62).

* Corresponding author. Mailing address: Department of Chemical and Biochemical Engineering, University of California, Irvine, Irvine, CA 92697-2575. Phone: (714) 824-3147. Fax: (714) 824-2541. E-mail: tkwood@uci.edu.

† Present address: Department of Chemical Engineering, University of Texas at Austin, Austin, TX 78712-1062.

TABLE 1. Bacteria and plasmids

Strain or plasmid	Genotype and/or phenotype[a]	Source (reference)
Strains		
E. coli XL1-Blue	F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*/*recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r_k^-m_k^+$) *supE44 relA1 lac*	Promega (10)
E. coli CC118(λ*pir*)	λ*pir* lysogen Δ(*ara-leu*) *araD* Δ(*lacX74*) *phoA20 thi-1 rpoB argE* (am) *recA1*; recipient of pCNB derivatives	V. de Lorenzo (22)
E. coli S17-1(λ*pir*)	λ*pir recA thi pro hsdR*$^-$*M*$^+$ *RP4*:2-Tc:Mu:Km Tn*7*Tp^{r}Sm^r; mobilizing strain for pCNB derivatives	V. de Lorenzo (14)
B. cepacia $PR1_{23C}(TOM_{23C})$	Km^r *Pcnst*::*tomA*$^+$*B*$^+$; constitutive expression of *tomA*$^+$*B*$^+$	M. Shields (47)
P. fluorescens 2-79	Wild type (NRRL B-15132)	USDA (61)[b]
P. fluorescens 2-79TOM	2-79 with chromosomal mini-Tn*5* *Pc*::*tomA*$^+$*B*$^+$ insertion	This study
P. fluorescens 2-79TOM-S	2-79 with chromosomal mini-Tn*5* *nahR*/*Psal*::*tomA*$^+$*B*$^+$ insertion	This study
Plasmids		
pUC18Not	Ap^r; identical to pUC18 but with *Not*I/MCS of pUC18/*Not*I as new MCSC[c]	V. de Lorenzo (15)
pMS64	Ap^r; *Pcnst*::*tomA*$^+$*B*$^+$; *E. coli* origin	M. Shields (47, 48)
pCNB4	Km^r Ap^r; *ori* R6K *mobRP4* pUT/mini-Tn*5* *nahR*/*Psal*	V. de Lorenzo (15)
pLANT1	Ap^r; *Pcnst*::*tomA*$^+$*B*$^+$ inserted into pUC18Not	This study
pLANT3	Km^r Ap^r; *Pcnst*::*tomA*$^+$*B*$^+$ inserted into pCNB4	This study
pLANT4	Km^r Ap^r; *tomA*$^+$*B*$^+$ inserted into pCNB4 under *nahR*/*Psal* control	This study

[a] *Pcnst* refers to the constitutive Tom promoter created by mutation of the phenol-inducible promoter of *tomA*$^+$*B*$^+$ (47).
[b] USDA, U.S. Department of Agriculture.
[c] MCSC, multiple cloning site.

Because of the ecological risk posed by soil and water contaminated with TCE, the toluene *o*-monooxygenase (Tom) genes of *Burkholderia cepacia* G4 have been cloned to utilize this enzyme for TCE degradation (48). Treatment of TCE by bacteria expressing Tom is advantageous because TCE is degraded primarily to CO_2 and Cl^- in vivo (30, 37). *B. cepacia* G4 and *B. cepacia* G4 $PR1_{23}(TOM_{23C})$ (referred to below as $PR1_{23C}$) have been used in various bioreactors to treat TCE-contaminated air and water (17, 49, 54). The Tom DNA sequence indicates that it consists of six genes (*tomA012345*) (46), and this enzyme is a member of the *mmo* family of monooxygenases (11, 23, 38) since it contains a 211-kDa hydroxylase with a catalytic oxygen-bridged binuclear iron center, a 40-kDa reductase, a 10.4-kDa protein involved in electron transfer between the hydroxylase and reductase, and a 12-kDa protein which may be responsible for adding iron to the hydroxylase apoenzyme (40). To produce $PR1_{23C}$, the wild-type phenol-induced promoter of *tomA* was mutated by transposon mutagenesis, and this strain reverted to constitutive expression of Tom (47).

Below we describe effective rhizoremediation of TCE with a recombinant root-colonizing bacterium introduced into the soil by planting wheat seeds coated with the bacterium. A TCE-degrading, rhizosphere-competent bacterium was constructed from *P. fluorescens* 2-79 by chromosomal insertion of the $PR1_{23C}$ genes encoding constitutive expression of Tom (48) with an integration vector developed by de Lorenzo et al. (15). *P. fluorescens* 2-79 colonizes wheat roots (61) better than many other known wheat root colonizers (35) and has been used to coat wheat seeds to inhibit disease-causing fungi, such as *Gaeumannomyces graminis* var. *tritici* and *Tilletia laevis*, through production of phenazine-1-carboxylic acid (7, 21, 34). Sealed bacterium-plant-soil microcosms were utilized to remove 63% of the initial TCE after 4 days, demonstrating the feasibility of rhizoremediation for the treatment of TCE-contaminated surface and near-surface soils (depth, 0 to 3 m), such as those found at the El Toro Marine base (El Toro, Calif.) (53) and the Miscellaneous Chemicals Basin (Aiken County, S.C.) (60).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacteria and plasmids used are listed in Table 1. *P. fluorescens* 2-79, the recombinant strains, and $PR1_{23C}$ were cultured in 250-ml Erlenmeyer flasks containing 25 ml of Luria-Bertani (LB) medium (43) at 30°C and shaken at 250 rpm (series 25 shaker; New Brunswick Scientific Co., Edison, N.J.). *Escherichia coli* strains were similarly cultured in LB medium but were shaken at 37°C. Bacteria containing *Psal*::*tomA*$^+$*B*$^+$ were induced with 2.0 mM sodium salicylate (Fisher Scientific, Tustin, Calif.).

Transformed *E. coli* cells were selected by using either MacConkey agar plates (Difco Laboratories, Detroit, Mich.) (31) containing 100 μg of ampicillin per ml or LB agar plates containing 100 μg of ampicillin per ml and 50 μg of kanamycin per ml and were incubated at 37°C. *P. fluorescens* transconjugants were selected on M9 minimal salts (42) agar plates containing 400 μg of kanamycin per ml and incubated at 30°C. Quantification of cell densities on roots was performed by using King's medium B (KMB) (26) agar plates (2% Proteose Peptone No. 3 [Difco], 0.15% K_2HPO_4 [Fisher], 0.15% $MgSO_4 \cdot 7H_2O$ [Fisher], 1% glycerol [Fisher], 1.5% Bacto Agar [Difco]) with or without 400 μg of kanamycin per ml.

Wheat cultivation and bacterial inoculation. Cavalier winter wheat seeds (Stover Seed Co., Los Angeles, Calif.) were sterilized with hydrogen peroxide as described by Somasegaran (52) and were germinated before bacterial inoculation and planting.

Sterilized wheat seeds were germinated by incubating them on wet sponges. The sponges were sterilized by soaking them in a 2.5% sodium hypochloride solution for 30 min, rinsed, and individually autoclaved in a foil-covered beaker containing water (the sponges were not submerged). The sterilized wheat seeds were placed on the wet sponges and incubated for 48 h at room temperature in the dark.

Germinated seeds were inoculated by placing the seeds in an open petri plate under a laminar flow hood. Then 10 ml of an overnight bacterial culture grown in LB medium (final A_{600}, 1.4 to 1.6) was transferred to the seed-containing plate and allowed to dry under the hood for 4 h. A total of 10 to 15 seeds were transferred by using sterile forceps to microcosms or were planted in plastic pots (with drainage holes) containing 75 g of Perma-Gro potting soil (The Garden Grow Co., Independence, Oreg.) and 50 ml of sterile tap water. The potted plants were then placed in an enclosed space 2 ft under a 60-W Gro & Sho plant light bulb (General Electric, Cleveland, Ohio) which was illuminated for 15 h each day and were watered with 25 ml of sterile tap water every 48 h.

Microcosms were created in modified 64-oz Qorpak clear wide-mouth bottles with Teflon-lined closures (Fisher). Each bottle was modified by adding a glass nipple to a 0.25-in. hole on the side of the bottle 17 cm from the bottom. The nipple permitted the attachment of a glass sampling port apparatus with Viton tubing. Between the nipple and glass sampling port was a Teflon stopcock which was opened when the headspace was sampled for TCE. A 60-ml disposable syringe (Becton Dickinson, Franklin Lakes, N.J.) connected to the other end of the sampling port with Luer Lok fittings was used to fill the sampling port with gas from the primary microcosm chamber.

Wheat was planted in the microcosms (100 to 175 seeds per microcosm), each of which contained 400 g of potting soil and 200 ml of sterile tap H_2O, and was

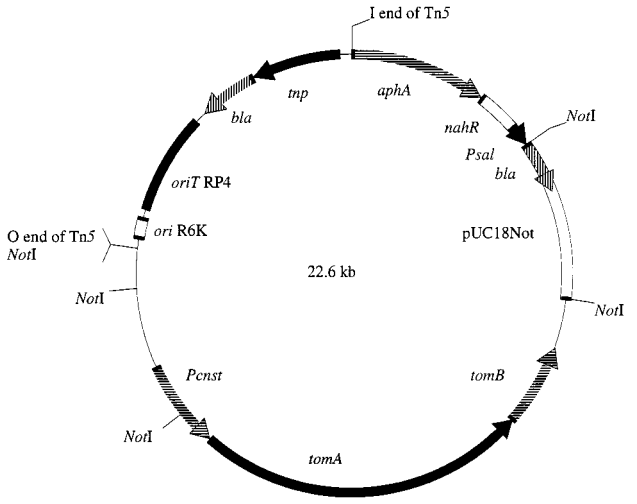

FIG. 1. pLANT3 plasmid restriction map. *Psal*, salicylate-induced promoter; *Pcnst*, constitutive promoter of *B. cepacia* $PR1_{23}(TOM_{23C})$; *tomA*, Tom gene; *tomB*, catechol 2,3-dioxygenase gene; *tnp*, transposase gene; *bla*, β-lactamase gene which confers ampicillin resistance; *aphA*, aminoglycoside 3′-phosphotransferase A gene from Tn*903* which confers kanamycin resistance; *ori* R6K, R6K origin of replication; *oriT* RP4, mobilizing region. The region between the I and O ends of Tn*5* is transposed.

cultivated at room temperature; the plants were exposed to 14 h of light per day. After 6 to 8 days of plant growth, TCE and $CHCl_3$ (used as a gas chromatography internal standard) were added to the microcosms by pipetting in a 50-ml solution containing 6.4 μmol of TCE and 6.4 μmol of $CHCl_3$ in water, which resulted in final concentrations of 1.7 μM (assuming that all of the TCE was in the gas phase in an empty 3.85-liter microcosm) or 128 μM (assuming that all of the TCE was in the 50 ml of liquid). Since the Teflon-taped threads of the microcosms were quickly capped after TCE addition, no additional water was added during the remainder of the experiment.

To determine whether TCE added to a microcosm at the soil surface was accessible to bacteria throughout the 7- to 8-cm soil depth, two separate microcosms were prepared, each containing only soil. TCE was introduced into one microcosm by evenly pipetting the 50 ml of TCE-$CHCl_3$ solution over the soil surface. TCE was injected with a syringe into the bottom of the other microcosm. After the microcosms were capped, the headspaces were monitored through the sampling ports to determine if the TCE in the two microcosms equilibrated to the same concentration.

Plasmid construction, electroporation, and conjugation. Before we created the final pLANT3 (constitutive) and pLANT4 (salicylate-induced) general constructs which allowed integration into gram-negative bacteria, an 11.3-kb DNA fragment containing $tomA^+B^+$ ($tomB^+$ encodes catechol 2,3-dioxygenase) and its constitutive promoter (*Pcnst*) were first isolated from pMS64 (48) by using an *Eco*RI digest; these genes were inserted into the unique pUC18Not *Eco*RI site (creating pLANT1), yielding *Not*I sites flanking the $tomA^+B^+$ locus, by using accepted molecular biology techniques (43). These sites allowed insertion into the unique *Not*I site of the pCNB4 integration vector after partial digestion (two additional *Not*I sites are located within the *tom* locus).

pLANT3 (22.6 kb, constitutive expression of Tom) (Fig. 1) was obtained by ligating the complete linearized 14.0-kb pLANT1 vector cut at the *Not*I site located upstream of the constitutive $tomA^+B^+$ promoter into pCNB4. The orientation of this 14.0-kb insert is opposite that of *Psal*, the salicylate-induced promoter in pCNB4. pLANT4 (20.0 kb, salicylate-induced expression of Tom) was obtained by ligating the 11.3-kb *Not*I fragments of pLANT1 (cut at the *Not*I sites located within and upstream of *Pcnst*) into pCNB4, which yielded Tom expression under salicylate control (*Psal*). The plasmids were verified by their restriction enzyme (*Sma*I, *Eco*RI, and *Not*I) digestion patterns by using horizontal agarose and vertical polyacrylamide gel electrophoresis (43).

Electroporation of pLANT1 into *E. coli* XL1-Blue and electroporation of pLANT3 (or pLANT4) into both *E. coli* CC118(λ*pir*) (used to isolate the pLANT3 and pLANT4 transformants) and *E. coli* S17-1(λ*pir*) (used as the donor strain for conjugating vectors into *P. fluorescens* 2-79) were performed by using 0.1-cm cuvettes with a Gene Pulser/Pulse Controller (Bio-Rad Laboratories, Hercules, Calif.) at 15 kV/cm, 25 μF, and 200 Ω, yielding time constants of 4.3 to 4.6 s. Electrocompetent cells were prepared by using the procedure of Smith and Iglewski (51). MacConkey-ampicillin agar plates were used to select white colonies as putative pLANT1 transformants, which were screened as brown colonies on plates containing LB medium supplemented with ampicillin and kanamycin.

The pCNB4-based plasmids (pLANT3 and pLANT4) function as single-event, chromosomal-integration vectors because the transposase gene is located outside the transposition region, and the vectors replicate only in *E. coli* strains that contain λ*pir* (λ phage carrying the *pir* gene, essential for R6K plasmid replication) (15). Therefore, both pLANT3 and pLANT4 were transferred to *P. fluorescens* 2-79 through biparental conjugation of either *E. coli* S17-1(λ*pir*)/pLANT3 or *E. coli* S17-1(λ*pir*)/pLANT4 with the root-colonizing recipient hosts by filter mating by using a modification of the methods of Brazil et al. (6) and Kim and Wood (25) in which 1.0-ml portions of both donor and recipient cells (each grown to an A_{600} of 1) were vortexed for 10 s in 10-ml disposable Falcon culture tubes (Fisher). The vortexed cells were then transferred to a sterile, 10-ml disposable syringe with a Luer Lok fitting (Fisher) and filtered through a sterile 0.45-μm-pore-size Supor-450 membrane filter (Gelman Sciences) in a 25-mm filter holder with Luer Lok fittings. The membrane was then removed and placed cell side up on an LB agar plate. Following a 16-h incubation at 30°C, the membrane was transferred, by using sterile forceps, to the bottom half of a sterile petri plate. The cells were washed off the membrane with 3.0 ml of sterile 100 mM sodium phosphate buffer (pH 7), and 100-μl aliquots of the resultant wash were plated onto M9 minimal salts agar plates supplemented with kanamycin.

The recombinant *P. fluorescens* strains that expressed Tom constitutively were also screened by using a colorimetric plate assay originally developed for soluble methane monooxygenase detection (19) and based on the reaction of 1-naphthol (enzymatically oxidized from naphthalene by Tom) with *o*-dianisidine (59). The transformant-containing M9 minimal salts agar-kanamycin plates were inverted, and several naphthalene crystals were added to each inverted cover; then the plates were incubated at 30°C for 15 min. A fresh 5-mg/ml *o*-dianisidine solution was lightly sprayed on the cell colonies, after which positive, Tom-expressing colonies turned purple. The *o*-dianisidine screening assay was employed because the naturally yellow *P. fluorescens* colonies interfered with a trifluoromethylphenol-based assay, in which $tomA^+B^+$-expressing colonies turn yellow (47). A *P. fluorescens* strain containing integrated $tomA^+B^+$ under the constitutive promoter was designated *P. fluorescens* 2-79TOM, and a *P. fluorescens* strain containing integrated $tomA^+B^+$ under control of the salicylate-induced promoter was designated *P. fluorescens* 2-79TOM-S.

Tom activity assay. By using a gas chromatograph, Tom activity was measured by determining the initial rate of TCE degradation (18). Cell cultures (grown to an A_{600} of approximately 1) were washed with 0.1 M potassium phosphate buffer (pH 7) by centrifugation at 10,000 × *g* at 25°C for 1 min by using a type JA-20 rotor (Beckman Instruments, Fullerton, Calif.) in a model J2-21 centrifuge. The cell density was measured after resuspension in the same volume of 0.1 M potassium phosphate buffer (pH 7.0).

Samples were prepared in either duplicate or triplicate with 60-ml serum vials, each containing 10 ml of resuspended cells. After the vials were capped with Teflon-coated silicone septa (Wheaton, Millville, N.J.) and aluminum crimp seals, 0.01 M TCE (Fisher) and 0.01 M chloroform (Fisher) in *N*,*N*-dimethylformamide (Fisher) were added by using a 10-μl liquid syringe (Hamilton, Reno, Nev.) to final concentrations of 10 μM (assuming that all of the TCE and all of the $CHCl_3$ were dissolved in the liquid). The inverted vials were shaken on a model KS125 shaker (IKA, Munich, Germany) at 350 rpm and room temperature for 5 min before 25-μl headspace samples were removed at 5-min intervals with a 50-μl gas-tight syringe (Hamilton). The gas samples were injected into a model 5980 Series II gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with an electron capture detector and a column packed with 0.1% AT-1000 on 80/100 Graphpac (Alltech, Deerfield, Ill.) (column temperature, 140°C; injection port and detector temperature, 190°C; 30 ml of N_2 per min). Retention times of 0.6 and 1.4 min were observed for $CHCl_3$ and TCE, respectively. Because $CHCl_3$ is degraded by neither *P. fluorescens* 2-79 (data not shown) nor $PR1_{23C}$ (33), $CHCl_3$ was used as an internal standard against which the TCE peak areas were normalized. To normalize the initial TCE degradation rates, the total cell protein concentration (0.20 ± 0.08 mg/ml) was determined after NaOH treatment of triplicate shake flask cell samples (optical density, 1) by using a protein assay kit (Bio-Rad Laboratories) based on the Bradford method (5) and bovine serum albumin standards.

Microcosm headspace samples were examined by first opening the stopcock connecting the bottle to the sampling port. To circulate the microcosm air without air loss, the attached syringe plunger was quickly pumped six times, extended for 30 s, and then depressed and held for 30 s before a gas sample was withdrawn for each measurement. The first measurement of TCE concentration (based on duplicate sample injections) was obtained 3 h after TCE addition, and samples were withdrawn once per day.

The extent-of-TCE degradation experiments were conducted similarly except that bacterial cultures were washed in 10-ml portions of fresh LB media (instead of phosphate buffer) and incubated with either 10 or 50 μM TCE (assuming that all of the TCE was dissolved in the liquid) in duplicate vials. The vials were shaken for 18 h at 350 rpm and room temperature, after which a syringe was used to add 10 ml of 10 μM $CHCl_3$ in pentane to each serum vial. The pentane-containing vials were shaken at room temperature for 5 min before the septa were removed and 2.0 ml of each pentane extract was transferred to a 2.0-ml screw-cap vial with a Teflon-silicone seal. The TCE remaining was measured by injecting 1.0-μl portions of the pentane extracts into the gas chromatograph and using the same conditions as those used for the headspace analysis. After the

TABLE 2. Shake flask extents of TCE degradation by Tom after 18 h, shake flask initial TCE degradation rates (first 5 min, 10 μM TCE), specific growth rates, and root colonization in nonmicrocosm studies

Sample	% of TCE removed[a]	Initial TCE degradation rate (nmol/min · mg of cells)	Specific growth rate in LB medium (h^{-1})	Root colonization (10^6, CFU/cm of root)
P. fluorescens 2-79 (negative control, no *tom*)	2	0.0	0.77	4
P. fluorescens 2-79TOM (constitutive Tom)	97	1.1	0.78	3
P. fluorescens 2-79TOM-S (uninduced, no salicylate)	14	0.0	ND[b]	ND
P. fluorescens 2-79TOM-S + 2 mM salicylate	99	2.6	0.77	3
B. cepacia $PR1_{23}(TOM_{23C})$ (constitutive, positive control, non-root colonizer)	98	2.5	0.86	0.04

[a] The initial TCE concentration was 10 μM.
[b] ND, not determined.

TCE peak areas were normalized against the $CHCl_3$ peak areas, the concentration of TCE remaining in each sample was determined from a TCE standard curve made by using known concentrations of TCE (0, 2, 4, 6, 8, and 10 μM TCE) in pentane. The pentane extraction efficiency was close to 100% since the TCE concentration obtained after we extracted the negative controls (containing known amounts of TCE added to fresh LB medium without bacteria) was virtually the same as that determined for the standardization samples in which the same amount of TCE was added directly to pentane.

Stability of Tom expression. The stability of Tom expression in *P. fluorescens* 2-79TOM and 2-79TOM-S was evaluated by measuring initial TCE degradation rates in the presence of 10 μM TCE (assuming that all of the TCE was in the liquid) after serial dilutions. The bacteria were grown in duplicate LB medium liquid cultures without kanamycin. Cultures of *P. fluorescens* 2-79TOM-S were supplemented with 2 mM sodium salicylate (Fisher) for full induction of Tom throughout the experiment. Serial dilutions were prepared every 24 h by inoculating 25 ml of fresh LB medium with 2 μl of the previous culture. A 10-ml sample of each 1-day-old culture was washed and resuspended in phosphate buffer, and the initial TCE degradation rate was determined by using serum vials as described above by measuring the TCE concentration in the headspace every 5 min and then using the initial degradation rate.

Specific growth rate determinations. The maximum specific growth rates of *P. fluorescens* 2-79TOM and 2-79TOM-S were determined by culturing each strain in duplicate flasks containing 50 ml of LB medium without antibiotics (the medium was supplemented with 2 mM sodium salicylate for *P. fluorescens* 2-79TOM-S). Each flask was inoculated with 20 μl of an overnight LB medium culture, and once the cultures reached an A_{600} of 0.05, the absorbance was measured every 5 min until the cells reached the stationary phase.

Root colonization measurements. After either 14 days of growth in microcosms or 7 days of growth in pots, root samples were obtained by cutting and suspending 2- to 4-cm sections of wheat roots in 2.0 ml of sterile 100 mM potassium phosphate buffer (pH 7). Each 2.0-ml root suspension was sonicated with a model 300 Sonic Dismembrator (Fisher) for 30 s by using a microtip (35% power) and then serially diluted with sterile 100 mM potassium phosphate buffer (pH 7). Dilutions were plated onto either KMB (for *P. fluorescens* 2-79 and $PR1_{23C}$) or KMB containing 400 μg of kanamycin per ml (for *P. fluorescens* 2-79TOM and 2-79TOM-S), and the fluorescent colonies were counted.

RESULTS

Construction of $tomA^+B^+$ integration plasmids and Tom-expressing strains. Two plasmids, pLANT3 (Fig. 1) and pLANT4, were constructed to facilitate the chromosomal integration of $tomA^+B^+$ and allow constitutive and inducible expression of Tom, respectively. *P. fluorescens* 2-79 was conjugated with the donor *E. coli* (containing either pLANT3 or pLANT4), which created recombinant, root-colonizing bacteria that possessed $tomA^+B^+$. The $tomA^+B^+$ genes appeared to be integrated into the chromosome rather than into an indigenous plasmid based on a restriction digestion analysis of plasmid mini-preps of *P. fluorescens* 2-79TOM and 2-79TOM-S cultures which did not yield any *Not*I fragments (which would have arisen if transposition into a plasmid occurred). Recombinant *E. coli* cultures that expressed Tom (i.e., contained pLANT1 or pLANT4) turned the LB medium brown (typical of *E. coli* strains containing pMS64 [unpublished data]) after approximately 16 h. *P. fluorescens* strains that expressed Tom turned LB medium bright yellow, whereas recombinant and wild-type *Rhizobium meliloti* and wild-type *E. coli* and *P. fluorescens* cultures remained white. Interestingly, even uninduced strains that contained $tomA^+B^+$ under *Psal* control turned the LB medium cultures brown [*E. coli* CC118(λ*pir*)/pLANT4 and *E. coli* S17-1(λ*pir*)/pLANT4] or bright yellow (*P. fluorescens* 2-79TOM-S).

To verify that Tom was actively expressed, putative *P. fluorescens* clones were examined to determine their extent of TCE degradation. TCE degradation was observed in both the constitutive and salicylate-induced recombinant *P. fluorescens* strains (Table 2), but not in the wild-type strain. The TCE degradation measurements show that the recombinant *P. fluorescens* strains degraded TCE to levels comparable (more than 95% of the initial TCE removed) to the levels obtained with $PR1_{23C}$ (the strain from which $tomA^+B^+$ was isolated). Additionally, expression of $tomA^+$ in *P. fluorescens* 2-79TOM-S was shown to be controlled by the salicylate promoter as minimal TCE degradation was observed in the uninduced samples (less than 15% of the TCE was removed).

Tom expression was not observed in the salicylate-induced and constitutive constructs created from *R. meliloti* 102F34, which colonizes alfalfa (4). The levels of catechol 2,3-dioxygenase activity resulting from $tomB^+$ expression in *P. fluorescens* 2-79TOM and *R. meliloti* 102F34TOM were 48 and 69%, respectively, of the level in $PR1_{23C}$ (11.4 nmol/optical density unit · min).

Characterization of recombinant *P. fluorescens* strains. When shake flask cultures were used, the maximum specific growth rates of Tom-expressing *P. fluorescens* 2-79TOM and 2-79TOM-S were found to be identical to the maximum specific growth rate of the wild-type host (Table 2). In addition, the salicylate-induced organism *P. fluorescens* 2-79TOM-S was shown to degrade TCE at a rate equivalent to the rate observed with $PR1_{23C}$ (2.5 nmol/min · mg of protein) and more than twice the rate observed with the constitutive organism *P. fluorescens* 2-79TOM (1.1 nmol/min · mg of protein). Furthermore, the ability of the recombinants to colonize wheat roots after 1 week of plant growth was relatively unchanged compared with the ability of the wild-type organism *P. fluorescens* 2-79. Hence, the physiological effects of insertion of the foreign genes into the chromosome were minimal. Although the constitutive strain degraded TCE at a lower rate than the inducible construct, *P. fluorescens* 2-79TOM was selected for TCE degradation studies in the microcosms because extents of TCE degradation similar to the induced *P. fluorescens* 2-79TOM-S extent of degradation were observed without a homogeneously distributed salicylate inducer in the soil. The *P. fluorescens* strains were also shown to colonize wheat roots at densities 100 times greater than those of $PR1_{23C}$ (which is not known to colonize wheat).

Stability of Tom expression. The long-term stability of TCE degradation by Tom was determined by measuring the initial TCE degradation rates of *P. fluorescens* 2-79TOM cultures

prepared each day by serially diluting the preceding culture (without antibiotics). Expression of Tom by *P. fluorescens* 2-79TOM was very stable, and TCE was degraded at a constant rate (1.0 ± 0.3 nmol/mg of protein · min) over a period of 37 days. Assuming that there was 8 h of exponential growth per day, the constitutively expressed strain was stable for more than 280 cell generations. A similar experiment performed with *P. fluorescens* 2-79TOM-S (continuously cultured and induced in the presence of 2 mM sodium salicylate without antibiotics) also resulted in constant TCE degradation rates for 92 generations before the experiment was arbitrarily ended.

In situ TCE degradation in microcosms. The degradation of TCE with wheat colonized by TCE-degrading bacteria was evaluated by using microcosms containing soil planted with bacterium-coated wheat seeds. TCE was observed to be relatively dispersed throughout the microcosms, as TCE added to either the top or bottom of the soil in the microcosms equilibrated to the same headspace concentration after less than 24 h. Therefore, in the microcosm experiments, the volatile organic compound was accessible to the TCE-degrading bacteria throughout the soil.

Data for TCE degradation in individual microcosms are shown in Table 3. Microcosms planted with wheat inoculated with *P. fluorescens* 2-79TOM had degraded an average of 31% of the TCE after 1 day and 51% after 2 days, and the degradation plateaued at 63% TCE removal after 4 days. Over the same 4-day period, negative control microcosms planted with wild-type *P. fluorescens* 2-79-inoculated wheat, uninoculated wheat, or sterile soil exhibited an average 9% decrease in the initial TCE concentration. Hence, negligible amounts of TCE and $CHCl_3$ were adsorbed by the soil or wheat, and the wild-type bacterium did not degrade TCE.

TABLE 3. TCE microcosm data and parameters

Sample[a]	Extent of TCE degradation (% of TCE removed)	Avg TCE degradation (% of TCE removed)	Length of expt (days)	Root colonization (10^5, CFU/cm of root)	Initial degradation rate (nmol/day · plant)[b]	Length of plant growth before TCE was added (days)	No. of seeds
Uninoculated microcosm 1	19	9 ± 8[c]	12	NA[d]	0	5	100
Uninoculated microcosm 2	4		9	NA	2	5	140
Uninoculated microcosm 3	5		6	NA	2	7	130
Sterile soil microcosm 1	3	4 ± 1	6	NA	0	NA	NA
Sterile soil microcosm 2	5		6	NA	0	NA	NA
2-79 microcosm 1	26	13 ± 7	11	ND[e]	7	7	110
2-79 microcosm 2	7		6	7 ± 2	1	7	160
2-79 microcosm 3	16		7	16 ± 8	5	7	160
2-79 microcosm 4	15		6	11 ± 4	2	7	160
2-79 microcosm 5	6		6	6 ± 5	1	7	160
2-79 microcosm 6	10		6	5 ± 2	0	7	160
2-79TOM microcosm 1	53	63 ± 11	6	ND	12	8	150
2-79TOM microcosm 2	67		6	ND	22	8	150
2-79TOM microcosm 3	85		6	ND	39	8	150
2-79TOM microcosm 4	48		7	0.7 ± 0.03	15	6	160
2-79TOM microcosm 5	65		7	11 ± 9	26	6	160
2-79TOM microcosm 6	63		7	2 ± 1	24	6	160
2-79TOM microcosm 7	62		7	10 ± 2	15	6	175
2-79TOM microcosm 8	71		7	9 ± 4	14	6	175
2-79TOM microcosm 9	55		7	5 ± 4	19	6	175

[a] Uninoculated microcosms contained sterilized soil and wheat; 2-79 microcosms contained sterilized soil, wheat, and *P. fluorescens* 2-79; and 2-79TOM microcosms contained sterilized soil, wheat, and *P. fluorescens* 2-79TOM. The initial TCE concentration in all microcosms was 128 μM (assuming that all of the TCE was in 50 ml of water in each microcosm jar).

[b] The initial TCE degradation rate was determined by determining the difference between the TCE concentrations in the headspace obtained 3 and 24 h after TCE was added.

[c] Mean ± standard deviation.

[d] NA, not applicable.

[e] ND, not determined.

DISCUSSION

Rhizoremediation of TCE was studied by using a recombinant *P. fluorescens* strain engineered to express Tom. By utilizing vectors derived from a general, chromosomal integration system originally developed by de Lorezno et al. (15), we created a root-colonizing strain that exhibits stable, constitutive Tom expression for more than 1 month. As demonstrated by the analogous results of Brazil et al. (6), the consistent expression observed shows that plasmid loss problems which can result in decreased degradation effectiveness may be overcome by integrating the expressed genes into the chromosome. Considering that extended treatment times are typically required for rhizoremediation, the stable, constitutive Tom expression exhibited by the recombinant *P. fluorescens* 2-79TOM and the relatively unchanged growth rate and root colonization characteristics (compared to wild-type *P. fluorescens* 2-79) suggest that *P. fluorescens* 2-79TOM is a microorganism well-suited for the study of TCE degradation in the rhizosphere. Besides colonizing the roots of a variety of plants, including wheat and peppers (32), this species also is advantageous because its growth (compared to the growth of *Pseudomonas putida*, for example) is relatively insensitive to TCE (55). Although *P. fluorescens* 2-79TOM colonizes wheat roots almost as effectively as the wild-type strain, a decline in rhizosphere fitness has been noted with other *P. fluorescens* strains constructed by chromosomal insertion. For example, using *P. fluorescens* R2f engineered by insertion of a *Bacillus* kanamycin marker gene (*npt*II) into the chromosome, van Elsas et al. determined that, although the recombinant and parental strains did not differ in growth rate and mixed-competition studies when they were performed in liquid cultures, the recombinant bacteria were outcompeted by the parental strain in rhizosphere studies in which the two bacteria were mixed (57). However, van Elsas et al. hypothesized that the decrease in rhizosphere competitiveness was a result of the NptII protein, rather than a gene disruption effect (57). This hypothesis is supported by the findings of Brazil et al., who observed that *P. fluorescens* F113pcb containing a *bph* chromosomal insert exhibits the same extent of colonization of beet roots as the parental strain (10^4 CFU/root) after 25 days in nonsterile soil coinoculated with *P. fluorescens* F113Rif (6). Therefore, the effect of the *tomA*$^+$*B*$^+$ insertion on the long-term survival of *P. fluorescens* 2-79TOM in competition with the wild-type strain and non-root-colonizing bacteria in the rhizosphere should be investigated. However, the relatively short-term microcosm experiments showed that TCE is effectively degraded by wheat roots colonized by this recombinant strain.

The decreasing rate of TCE degradation in the microcosms resulted from substrate concentration-dependent rate kinetics (18) and the nature of the unoptimized experimental apparatus. Primarily, the microcosms were sealed after TCE was added, thereby limiting the plants and TCE-degrading bacteria to a finite quantity of carbon dioxide, oxygen, and water. Ideally, the wheat would have utilized carbon dioxide produced by

the bacteria, while it provided oxygen for bacterial growth. However, the population of aerobic bacteria may not have been sufficient to sustain this cycle, and the wheat began to yellow and wilt 7 days after the microcosms were sealed. Oxygen limitation is particularly detrimental to TCE removal because oxygen is a necessary substrate of Tom (36). In addition, a closed environment does not allow for the release of unidentified gaseous by-products (observed in this study by their odor) resulting from plant and bacterial growth and/or TCE degradation. As these by-products accumulate, plant growth and bacterial degradation of TCE may be adversely affected.

TCE degradation may also be improved by enhancing root colonization by *P. fluorescens* 2-79TOM. The extent of root colonization by *P. fluorescens* 2-79 has been shown to increase with increasing cell populations on inoculated seeds (9). As observed with a similar *P. fluorescens* strain, this effect is more pronounced with wheat less than 3 weeks old (24). The number of cells associated with an inoculated seed may be increased by utilizing a carrier, such as methylcellulose. The long-term viability of *P. fluorescens* 2-79 in wheat coated with bacterial suspensions is also affected by the age at which *P. fluorescens* 2-79 is harvested, as well as the liquid medium in which the suspensions are created (50). Inoculating suspensions of the wheat-colonizing bacterium are less toxic to the wheat seedlings and are better able to withstand drying when the coating mixture is suspended in water rather than in metabolite-bearing, spent culture broth (similar to the cell suspension used in this study) (50).

As an approximation based on data obtained in this work with *P. fluorescens* 2-79TOM, each microcosm of 160 plants would be expected to degrade 8 nmol of TCE/(day · plant) or to completely remove 6.4 μmol of TCE after 5 days. This assumes that there is a sustained TCE degradation rate, that the cell density on colonized wheat roots is 10^5 cells/cm of root, that the average root length per plant is 20 cm, and that the TCE degradation rate is 1 nmol of TCE/min · mg of protein. This analysis, however, does not take into account TCE-degrading bacteria in the bulk soil (which are not directly associated with the colonized roots), which should result in higher rates of TCE degradation. The actual data show that under nonideal conditions (a closed microcosm system), an average of 20.6 nmol of TCE/(day · plant) is degraded after 1 day (Table 3), so our analysis was conservative.

The results show that rhizoremediation of TCE in surface soils is accomplished by a rhizosphere established in a defined microcosm by using wheat seeds coated with *P. fluorescens* 2-79TOM. 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 (e.g., trees). Therefore, further development of systems incorporating genetically engineered microorganisms promises to yield effective, low-cost rhizoremediation techniques.

ACKNOWLEDGMENTS

This work was supported by grant TS20616 from the University of California Toxic Substances Research and Teaching Program and by grant DAAH04-95-0310 from the Army Research Office.

We thank Sandra Cuevos, Katy Gao, Josh Gorham, and Leslie Recaido for help in establishing plant protocols and identifying restriction enzyme sites in plasmid pMS64.

REFERENCES

1. **Anderson, T. A., E. A. Guthrie, and B. T. Walton.** 1993. Bioremediation in the rhizosphere. Environ. Sci. Technol. **27:**2630–2636.
2. **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. Environ. Toxicol. Chem. **14:**2041–2047.
3. **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.
4. **Becker, A., A. Kleickmann, H. Kuster, M. Keller, W. Arnold, and A. Puhler.** 1993. Analysis of the *Rhizobium meliloti* genes *exoU*, *exoV*, *exoW*, *exoT*, and *exoI* involved in exopolysaccharide biosynthesis and nodule invasion: *exoU* and *exoW* probably encode glucosyltransferases. Mol. Plant Microbe Interact. **6:**735–744.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brazil, G. M., L. Kenefick, M. Callanan, A. Haro, V. de Lorenzo, D. N. Dowling, and F. O'Gara.** 1995. Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of *bph* gene expression in the rhizosphere. Appl. Environ. Microbiol. **61:**1946–1952.
7. **Brisbane, P. G., J. R. Harris, and R. Moen.** 1989. Inhibition of fungi from wheat roots by *Pseudomonas fluorescens* 2-79 and fungicides. Soil. Biol. Biochem. **21:**1019–1025.
8. **Bruckner, J. V., B. D. Davis, and J. N. Blancato.** 1989. Metabolism, toxicity, and carcinogenicity of trichloroethylene. Crit. Rev. Toxicol. **20:**31–50.
9. **Bull, C. T., D. M. Weller, and L. S. Thomashow.** 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. Phytopathology **81:**954–959.
10. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
11. **Byrne, A. M., J. J. Kukor, and R. H. Olsen.** 1995. Sequence analysis of the gene cluster encoding toluene-3-monooxygenase from *Pseudomonas pickettii* PKO1. Gene **154:**65–70.
12. **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 Microbiol. Ecol. **20:**79–89.
13. **Curl, E. A., and B. Truelove.** 1986. The rhizosphere. Springer-Verlag, Berlin, Germany.
14. **de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis.** 1993. Analysis of *Pseudomonas* gene products using *lacI*^{q}/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene **123:**17–24.
15. **de Lorenzo, V., S. Fernández, N. 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.
16. **Environmental Health Center.** 1997. Trichloroethylene (C_2HCl_3) chemical backgrounder. Environment Writer. http://www.nsc.org/ehc/ew/chems/trichlor.htm (22 January 1997).
17. **Folsom, B. R., and P. J. Chapman.** 1991. Performance characterization of a model bioreactor for the biodegradation of trichloroethylene by *Pseudomonas cepacia* G4. Appl. Environ. Microbiol. **57:**1602–1608.
18. **Folsom, B. R., P. J. Chapman, and P. H. Pritchard.** 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56:**1279–1285.
19. **Graham, D. W., D. G. Korich, R. P. LeBlanc, N. A. Sinclair, and R. G. Arnold.** 1992. Applications of a colorimetric plate assay for soluble methane monooxygenase activity. Appl. Environ. Microbiol. **58:**2231–2236.
20. **Halogenated Solvents Industry Alliance Inc.** 1996. Trichloroethylene. White Paper. http://www.hsia.org/trichloro.htm (16 May 1997).
21. **Hamdan, H., D. M. Weller, and L. S. Thomashow.** 1991. Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* 2-79 and M4-80R. Appl. Environ. Microbiol. **57:**3270–3277.
22. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
23. **Johnson, G. R., and R. H. Olsen.** 1995. Nucleotide sequence analysis of genes encoding a toluene/benzene-2-monooxygenase from *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. **61:**3336–3346.
24. **Juhnke, M. E., D. E. Mathre, and D. C. Sands.** 1989. Relationship between bacterial seed inoculum density and rhizosphere colonization of spring wheat. Soil Biol. Biochem. **21:**591–595.
25. **Kim, C. S., and T. K. Wood.** Electroporation of pink-pigmented methylotrophic bacteria. Appl. Biochem. Biotechnol., in press.
26. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44:**301–307.
27. **Kingsley, M. T., J. K. Fredrickson, F. B. Metting, and R. J. Seidler.** 1994. Environmental restoration using plant-microbe bioaugmentation, p. 287–

292. *In* R. E. Hinchee, A. Leeson, L. Semprini, and S. K. Ong (ed.), Bioremediation of chlorinated and polyaromatic hydrocarbon compounds. Lewis Publishers, Boca Raton, Fla.
28. **Lappin, H. M., M. P. Greaves, and J. H. Slater.** 1985. Degradation of the herbicide mecoprop [2-(2-methyl-4-chlorophenoxy)propionic acid] by a synergistic microbial community. Appl. Environ. Microbiol. **49:**429–433.
29. **Lugtenberg, B., A. van der Bij, G. Bloemberg, T. C. A. Woeng, L. Dekkers, L. Kravchenko, I. Mulders, C. Phoelich, M. Simons, I. Tikhonovich, L. de Weger, and C. Wijffelman.** 1997. Towards the molecular basis of plant root colonization by *Pseudomonas* bacteria. *In Pseudomonas* '97. VI International Congress on *Pseudomonas*: Molecular Biology and Biotechnology, Madrid, Spain.
30. **Luu, P. P., C. W. Yung, A. K. Sun, and T. K. Wood.** 1995. Monitoring trichloroethylene mineralization by *Pseudomonas cepacia* G4 PR1. Appl. Microbiol. Biotechnol. **44:**259–264.
31. **MacConkey, A. T.** 1908. Bile salt media and their advantages in some bacteriological examinations. J. Hyg. **8:**322–334.
32. **Marschner, P., and D. E. Crowley.** 1996. Root colonization of mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum*) by *Pseudomonas fluorescens* 2-79RL. New Phytol. **134:**115–122.
33. **McClay, K., B. G. Fox, and R. J. Steffan.** 1996. Chloroform mineralization by toluene-oxidizing bacteria. Appl. Environ. Microbiol. **62:**2716–2722.
34. **McManus, P. S., A. V. Ravenscroft, and D. W. Fulbright.** 1993. Inhibition of *Tilletia laevis* teliospore germination and suppression of common bunt of wheat by *Pseudomonas fluorescens* 2-79. Plant Dis. **77:**1012–1015.
35. **Milus, E. A., and C. S. Rothrock.** 1993. Rhizosphere colonization of wheat by selected soil bacteria over diverse environments. Can. J. Microbiol. **39:**335–341.
36. **Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard.** 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53:**949–954.
37. **Nelson, M. J. K., S. O. Montgomery, E. J. O'Neill, and P. H. Pritchard.** 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. Appl. Environ. Microbiol. **52:**383–384.
38. **Newman, L. M., and L. P. Wackett.** 1997. Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics, and turnover-dependent inactivation. J. Bacteriol. **179:**90–96.
39. **Ochsner, J. C., T. R. Blackwood, and W. C. Micheletti.** 1979. Status assessment of toxic chemicals: trichloroethylene. Publication EPA-600/2-79-210m. U. S. Environmental Protection Agency, Cincinnati, Ohio.
40. **Powlowski, J., J. Sealy, V. Shingler, and E. Cadieux.** 1997. On the role of DmpK, an auxiliary protein associated with multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600. J. Biol. Chem. **272:**945–951.
41. **Reddy, B. R., and N. Sethunathan.** 1983. Mineralization of parathion in the rhizosphere. Appl. Environ. Microbiol. **45:**826–829.
42. **Rodriguez, R. L., and R. C. Tait.** 1983. Recombinant DNA techniques: an introduction. Benjamin/Cummings Publishing, Menlo Park, Calif.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Schwab, A. P., M. K. Banks, and M. Arunachalam.** 1995. Biodegradation of polycyclic aromatic hydrocarbons in rhizosphere soils, p. 23–29. *In* R. E. Hinchee, D. B. Anderson, and R. E. Hoeppel (ed.), Bioremediation of recalcitrant organics. Battelle Memorial Institute, Columbus, Ohio.
45. **Scott, E. M., E. A. S. Rattray, J. I. Prosser, K. Killham, L. A. Glover, J. M. Lynch, and M. J. Bazin.** 1995. A mathematical model for dispersal of bacterial inoculants colonizing the wheat rhizosphere. Soil Biol. Biochem. **27:**1307–1318.
46. **Shields, M. S., and S. C. Francesconi.** August 1996. U.S. patent 5,543,317.
47. **Shields, M. S., and M. J. Reagin.** 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol. **58:**3977–3983.
48. **Shields, M. S., M. J. Reagin, R. R. Gerger, R. Campbell, and C. Somerville.** 1995. TOM, a new aromatic degradative plasmid from *Burkholderia* (*Pseudomonas*) *cepacia* G4. Appl. Environ. Microbiol. **61:**1352–1356.
49. **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, p. 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, Fla.
50. **Slininger, P. J., J. E. Van Cauwenberge, R. J. Bothast, D. M. Weller, L. S. Thomashow, and R. J. Cook.** 1996. Effect of growth culture physiological state, metabolites, and formulation on the viability, phytotoxicity, and efficacy of the take-all biocontrol agent *Pseudomonas fluorescens* 2-79 stored encapsulated on wheat seeds. Appl. Microbiol. Biotechnol. **45:**391–398.
51. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
52. **Somasegaran, P.** 1994. Handbook for rhizobia: methods in legume-rhizobium technology. H. J. Hoben, New York, N.Y.
53. **Southwest Division Naval Facilities.** 1993. Phase I remedial investigation. Publication 1002096A.SCO\93\JD. El Toro Marine Corps Air Station, El Toro, Calif.
54. **Sun, A. K., and T. K. Wood.** 1997. Trichloroethylene mineralization in a fixed-film bioreactor using a pure *Burkholderia cepacia* G4 PR1 culture expressing constitutively toluene ortho-monooxygenase. Biotechnol. Bioeng. **55:**674–685.
55. **Tørsløv, J.** 1993. Comparison of bacterial toxicity tests based on growth, dehydrogenase activity, and esterase activity of *Pseudomonas fluorescens*. Ecotoxicol. Environ. Saf. **25:**33–40.
56. **U. S. Environmental Protection Agency.** 1980. Ambient water quality criteria for trichloroethylene. Publication EPA 440/5-80-077. National Technical Information Service, Springfield, Ill.
57. **van Elsas, J. D., A. C. Wolters, C. D. Clegg, H. M. Lappin-Scott, and J. M. Anderson.** 1994. Fitness of genetically modified *Pseudomonas fluorescens* in competition for soil and root colonization. FEMS Microbiol. Ecol. **13:**259–272.
58. **Wackett, L. P., and D. L. Allan.** 1995. Comment on 'Bioremediation in the Rhizosphere.' Environ. Sci. Technol. **29:**551.
59. **Wackett, L. P., and D. T. Gibson.** 1983. Rapid method for detection and quantification of hydroxylated aromatic intermediates produced by microorganisms. Appl. Environ. Microbiol. **45:**1144–1147.
60. **Walton, B. T., and T. A. Anderson.** 1990. Microbial degradation of trichloroethylene in the rhizosphere: potential application to biological remediation of waste sites. Appl. Environ. Microbiol. **56:**1012–1016.
61. **Weller, D. M.** 1983. Colonization of wheat roots by a fluorescent pseudomonad suppressive to take-all. Phytopathology **73:**1548–1553.
62. **Westrick, J. J., J. W. Mello, and R. F. Thomas.** 1984. The groundwater supply survey. J. Am. Water Works Assoc. **76:**52–59.