Appl Microbiol Biotechnol (1995) 44:259–264

ORIGINAL PAPER

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Monitoring trichloroethylene mineralization by *Pseudomonas cepacia* G4 PR1

Received: 19 September 1994/Received revision: 17 January 1995/Accepted: 20 February 1995

Abstract To analyze the extent of mineralization of trichloroethylene (TCE) without disturbing an actively growing biofilm, a minimal growth medium was formulated that reduces the concentration of chloride ions to the extent that the chloride ions generated from TCE mineralization may be detected with a chloride-ion-specific electrode. By substituting chloride salts with phosphates and nitrates, a chloride-free minimal medium was produced that yields a specific growth rate for *Pseudomonas cepacia* G4 PR1 which was 93% of that in chloride-ion-containing minimal medium. Furthermore, TCE degradation by resting cell suspensions was similar in both media (85% of 75 μM TCE degraded in 6 h), and complete mineralization of TCE was slightly superior in the chloride-free minimal medium (77% compared to 60% of 75 μM TCE mineralized in 6 h). In addition, indole-containing, minimal-medium agar plates were developed to indicate the presence of the TCE-degrading enzyme toluene *ortho*-monooxygenase (fire-engine-red colonies) as well as to distinguish this enzyme from other TCE-degrading enzymes (toluene dioxygenase and toluene *para*-monooxygenase).

Introduction

Because of its widespread use and inadequate disposal, trichloroethylene (TCE) is the most-frequently-reported contaminant at hazardous waste sites on the National Priorities List of the U.S. Environmental Protection Agency (US EPA) (Little et al. 1988), and it threatens or contaminates the water supply of many communities (US EPA 1984). Bioremediation of TCE is an important emerging technology in that it is potentially the most cost-effective treatment alternative (McFarland et al. 1992; Winter et al. 1989). Unlike other treatment techniques, bioremediation is a final solution since it completely converts TCE into carbon dioxide and chloride ions (mineralization), avoids the transportation of hazardous materials, and is a publicly-acceptable technology.

To implement bioremediation effectively, it is important to monitor the activity of bacterial cells. However, commonly used media usually contain high concentrations of chloride ions that prevent *in situ* analysis of the extent of TCE mineralization (although recently a chloride-free minimal medium was used for the cultivation of *Streptomyces rochei* 303; (Golovleva et al. 1992). To determine the extent of TCE mineralization, cells are usually removed from the bioreactor, washed, and suspended in a phosphate buffer (before TCE is added) to remove background chloride ions (Nelson et al. 1987). To analyse TCE degradation without removing the biomass from a biofilter, a chloride-free minimal medium has been developed. With this medium, the TCE-degrading activity of *Pseudomonas cepacia* G4 PR1 in a biofilter may be determined without disturbing the biomass. *P. cepacia* G4 PR1 (Shields and Reagin 1992) is a transposon mutant of *P. cepacia* G4 (Nelson et al. 1987) that spontaneously reverted to constitutive expression of the TCE-degrading enzyme toluene *ortho*-monooxygenase (TOM) and has been shown to be effective in degrading TCE in a biofilter (Shields et al. 1994).

To confirm the presence of *P. cepacia* G4 PR1 and expression of its TCE-degrading enzyme TOM, indole agar plates were developed. Indole has been used in the past to indicate the presence of naphthalene dioxygenase of *P. putida* PpG7 (Ensley et al. 1983), of toluene dioxygenase of (TDO) *P. putida* NCIB11767 (Jenkins and Dalton 1985), and of toluene *para*-monooxygenase (T4MO) of *P. mendocina* KR1 (Yen et al. 1991); but this is the first report of indole plates being

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used for the TOM enzyme and the first comparison of the indole reaction with the three oxygenases. The minimal plates used gave a dark green-black for TDO, a light blue for T4MO, and a vivid red-orange for TOM. Hence, indole agar plates may be used to distinguish between TCE-degrading enzymes and bacteria.

Materials and methods

Chloride-ion-free medium

The M9/glucose minimal medium used for the agar plates and as a nutrient medium was that of Rodriguez and Tait (1983). The chloride-free minimal medium (MCl) was formulated by replacing the 0.1 mM $CaCl_2$ of M9 with 0.1 mM $Ca(NO_3)_2$; the 8.6 mM NaCl of M9 was omitted and the 18.7 mM NH_4Cl was replaced with 19 mM $(NH_4)_2HPO_4$. Hence, 1 l MCl consists of 4 g glucose, 0.024 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.246 g $MgSO_4 \cdot 7H_2O$, 7.0 g Na_2PO_4, 3.0 g KH_2PO_4, and 2.52 g $(NH_4)_2HPO_4$.

Bacterial strains and cultivation conditions

The TCE-degrading enzyme TOM of *P. cepacia* G4 (Folsom et al. 1990) was induced with phenol as the sole carbon source, and *P. cepacia* G4 PR1 (Shields and Reagin 1992) constitutively expresses TOM and is kanamycin-resistant. T4MO of *P. mendocina* KR1 (Yen et al. 1991) and TDO of *P. putida* F1 (Wackett and Gibson 1988) were induced with toluene as the sole carbon source. *E. coli* strain JM83/pMY401 (Yen et al. 1991) contains the *P. mendocina* KR1 T4MO *tmoABCDE* locus under control of the *lac* promoter, and *E. coli* strain JM109/pDTG601 (Zylstra and Gibson 1989) contains the *P. putida* F1 TDO *todC1C2BA* under control of the *tac* promoter. Strain JM109 was obtained from Promega Inc., Madison, Wis.

For induction of T4MO and TDO, seed cultures of *P. mendocina* KR1 and *P. putida* F1 were prepared by inoculation of 20 ml M9/glucose (0.4% w/v) in a 250-ml conical flask with a -85° C glycerol stock (15% v/v) and incubating overnight at 250 rpm and 30° C (Series 25, New Brunswick Scientific Co. Inc., Edison, NJ). A sample comprising 2% of this seed culture was used to inoculate 25 ml M9, with toluene (0.4% v/v) supplied in the vapor phase as the sole carbon source by placing 100 µl solvent in a small tube suspended from a rubber stopper above the medium. To induce TOM, *P. cepacia* G4 was grown overnight in M9/glucose (0.4% w/v) at 30° C by inoculating from a -85° C glycerol stock (15% v/v), then 1–2% of this seed culture was used to inoculate M9 medium with 5 mM phenol as the sole carbon source. For non-inducing conditions, the pseudomonads were cultured in Luria Bertani (LB) medium (with 50 µg/ml kanamycin added for *P. cepacia* G4 PR1). The *E. coli* strains were cultured in LB or M9/glucose medium with 40 µg/ml ampicillin added for strains harboring pDTG601 or pMY401 (no isopropyl thiogalactoside was required to induce expression of the cloned oxygenases).

Indole/agar indicator plates

The protocol of Rodriguez and Tait (1983) was followed for preparation of LB/agar and M9/glucose (0.4% w/v)/agar plates, and the LB/Fe/agar and M9/glucose/Fe/agar plates contained 40 µM filter-sterilized $FeSO_4$ (Whatman 0.2-µm cellulose nitrate filter). L-salts/agar plates contained 4 g/l glucose, 15 g/l agar, 0.25 g/l NH_4Cl, 0.015 g/l $CaCl_2$, 0.001 g/l $FeSO_4$, 0.2 g/l $MgSO_4$, 1.0 g/l $NaNO_3$, 0.04 g/l KCl, 0.21 g/l Na_2HPO_4, 0.09 g/l NaH_2PO_4, 0.168 mg/l $MoO_4 \cdot 2H_2O$, and 0.1 ml/10 000 × trace metal solution consisting of 1.0 g/l H_3BO_3, 1.0 g/l $MnSO_4 \cdot 5H_2O$, 1.0 g/l $ZnSO_4 \cdot 7H_2O$ (0.0005 g/l $CuSO_4$ was not added).

Owing to its pungent nature, indole (Aldrich Chemical, Milwaukee, Wis; I-340-8) was not dissolved in the agar; instead, roughly 5 mg indole crystals was added directly to the lid of the petri dish after 12–18 h of bacterial growth. It is important to avoid adding too much indole (roughly 25 mg) since it inhibits cell growth. However, too little indole (roughly 1 mg) leads to incomplete color development.

Extent of TCE degradation by *P. cepacia* G4 PR1 in M9 and MCl media

P. cepacia G4 PR1 was grown in M9/glucose and MCl media supplemented with 50 µg/ml kanamycin sulfate (United States Biochemical Corp., Cleveland, Ohio) by inoculating 200 ml in a 500-ml conical flask with approximately 50 µl -85° C glycerol stock and incubating overnight at 250 rpm and 30° C to reach an A_{600} of 1.0–3.0. The cells were washed twice with prewarmed (30° C) 0.1 M potassium phosphate buffer at pH 7 by centrifugation at 7000 rpm for 10 min at room temperature using a Beckman Instruments (Fullerton, Calif.) JA-20 rotor in a J2-21 centrifuge (roughly 5000 g), then the cells were resuspended in potassium phosphate buffer held at 30° C and the absorbance was adjusted to $A_{600} = 1.0$.

Triplicate samples were made by placing 5 ml resting cell suspension into 60-ml glass vials, which were sealed with Teflon-coated silicone septa (Wheaton, Millville, N.J.) and an aluminium crimp seal. A sample of 3.75 µl 0.1 M TCE (Fisher Scientific T341-500, Tustin, Calif.) dissolved in *N*,*N*-dimethylformamide (Fisher Scientific D131-1) in a no-head-space vial was then introduced into each vial using a 10-µl gas-tight syringe (Hamilton, Reno, Nev.) to make a final concentration of 75 µM (assuming all the TCE is dissolved in the liquid phase). Vials were inverted and shaken at 250 rpm and 30° C for the duration of the experiment, and 5-µl headspace samples were taken every 30 min with a 50-µl gas-tight syringe (Hamilton) and injected into a Varian 3600 gas chromatograph equipped with an electron-capture detector (Varian associates, Sunnyvale, Calif.). The gas chromatograph conditions have been described previously (Sun and Wood 1995) and resulted in a TCE peak retention time of 1.5–1.6 min. Negative controls consisted of both triplicate samples of TCE + phosphate buffer and TCE + heat-killed *P. cepacia* G4 PR1 cells adjusted to $A_{600} = 1.0$. The 100% baseline used to determine the extent of TCE degradation was determined from the average of the triplicate, first-hour, phosphate buffer + TCE samples.

Extent of TCE mineralization by *P. cepacia* G4 PR1 in M9 and MCl media

The suspension of resting *P. cepacia* G4 PR1 cells was prepared as in the TCE degradation experiments, and 10 ml of this cell suspension was placed in the 60 ml glass vials with aluminium crimp seals. A 7.5 µl sample of the 0.1 M TCE stock solution was introduced using a gas-tight syringe, and the vials were inverted and incubated at 250 rpm and 30° C. Every 2 h, triplicates of each sample were sacrificed by opening the seal, and the mineralization data were obtained by measuring the chloride ions generated with a model 13-620-519 chloride-ion-selective electrode and model 13-620-47 reference silver-chloride electrode (Fisher Scientific, Tustin, Calif.) attached to a Corning pH/ion analyser 350 (Corning Incorporated, Corning, N.Y.). Five NaCl standards (0.05–0.5 mM in potassium phosphate buffer at room temperature) were used for calibration, and 2% (v/v) 5 M sodium nitrate (ion-strengthening agent) was added before analyzing the 10 ml samples in a well-stirred 25-ml beaker. The negative controls consisted of 75 µM TCE in potassium

phosphate buffer and autoclaved *P. cepacia* G4 PR1 cells ($A_{600} = 1.0$) with 75 μM TCE in the same buffer. For all the samples, zero-time chlroide-ion concentrations were subtracted to obtain the actual change in the chloride ion concentration.

Specific growth rates and electroporation

The specific growth rates of *P. cepacia* G4 PR1 in M9/glucose minimal medium and in MCl medium (both supplemented with 50 μg/ml kanamycin) were determined by monitoring the absorbance at 600 nm on a Milton Roy (Rochester, N.Y.) Spectronic 20D spectrophotometer. Three 25-ml cultures in 250-ml conical flasks were shaken at 30° C in a rotary shaker at 250 rpm for growth in MCl medium whereas two flasks were used for M9/glucose medium.

To form JM109/pMY401, plasmid pMY401 was isolated from JM83/pMY401 using a plasmid mini-prep (Rodriguez and Tait 1983), and electroporated into JM109 using a Bio-Rad Laboratories (Hercules, Calif.) gene pulser/pulse controller following the manufacturer's protocol (conditions were 15 kV/cm, 25 μF, and 200 Ω). Colonies containing pMY401 were selected on LB/ampicillin (50 μg/ml) plates and verified by restricting the plasmid DNA obtained with *Nde*I, *Cla*I, *Hin*dIII, and *Bam*HI enzymes and checking the pattern with a model B1A horizontal minigel electrophoresis unit (Owl Scientific Plastics, Cambridge, Mass.)

Results

Extent of TCE degradation and mineralization in chloride-free medium

To determine whether *P. cepacia* G4 PR1 degrades TCE in the new MCl medium as well as in M9/glucose medium, suspensions of resting cells were used to degrade 75 μM TCE. As shown in Fig. 1, *P. cepacia* G4 PR1 resting cells degrade TCE equally well in both MCl and M9 media. After 6 h, *P. cepacia* G4 PR1 degraded 89% of the TCE in both MCl and M9 media. Similar results have been found for *P. cepacia* G4 PR1 in LB medium (93% removed in 6 h; Sun and Wood 1995).

To gauge whether MCl medium would be useful for monitoring TCE mineralization in a bioreactor, the extent of complete TCE mineralization (conversion to carbon dioxide and chloride ions) was determined by monitoring the increase in chloride ion concentration for resting cells (Nelson et al. 1987). As shown in Figs. 1, 2, *P. cepacia* G4 PR1 not only degrades 89% of the TCE but also mineralizes 77% of it as well. Similarly, 59% of the TCE was mineralized by *P. cepacia* G4 PR1 cells grown in M9 medium. TCE has been mineralized by *P. cepacia* G4 PR1 for over 8 weeks in a fixed-film bioreactor using MCl (unpublished results).

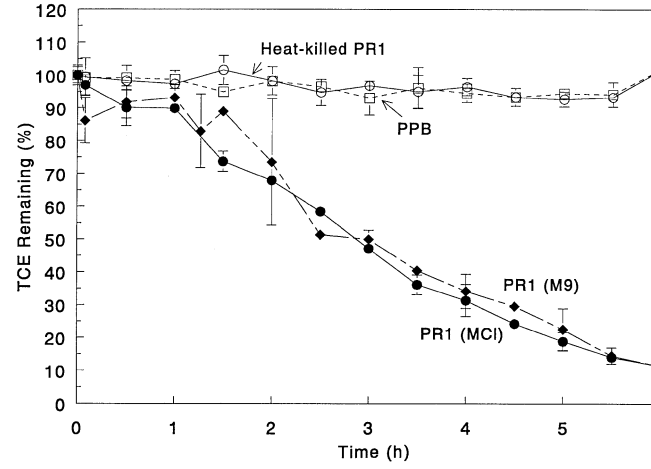

Fig. 1 Extent of trichloroethylene (TCE) (75 μM) degradation by *P. cepacia* G4 PR1 resting cells (*PR1*) in M9 and MCl minimal media as determined from gas chromatography measurements. Data represent the average of triplicate vials (error bars shown). *PPB* is potassium phosphate buffer used as a negative control

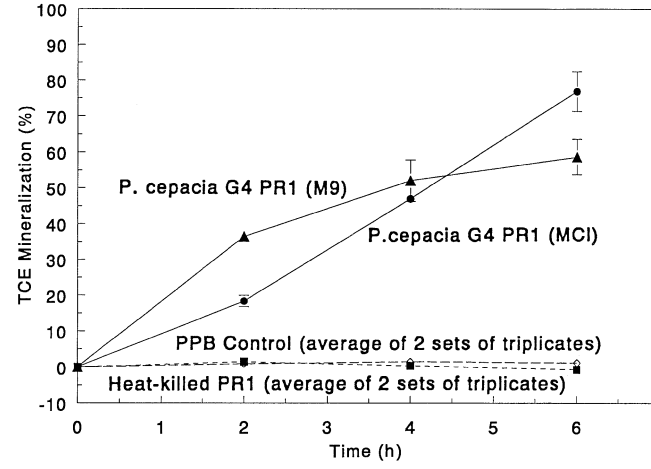

Fig. 2 Extent of TCE (75 μM) mineralization by *P. cepacia* G4 PR1 resting cells in M9 and MCl minimal media as determined from chloride-ion measurements. Data represent the average of triplicate vials (error bars shown). *PPB* is potassium phosphate buffer used as a negative control

Color formation on indole/agar plates

Good color development was achieved in 12–24 h by adding indole to the lid of the plates after the colonies had developed for 18–24 h. The pseudomonads grew best on the rich LB medium and slowest growth occurred on the L-salts plates. The best color development for *P. cepacia* G4 PR1, *P. cepacia* G4, and *P. putida* F1 occurred on L-salts plates (deep red/orange, light red/orange, dark green/black respectively) as shown in Table 1 and Fig. 3. The best color for *P. mendocina* KR1 was achieved with M9/glucose plates (light blue). There was no significant difference in color between pseudomonad cells that were grown under inducing conditions and under non-inducing conditions before plating (after plating, glucose or yeast extract was present as a carbon source). In addition, adding Fe^{2+} to LB and M9 plates did not make a significant difference but

Table 1 Comparison of colors of indole agar plates with various *Pseudomonas* strains. Bold entries indicate plates with best color development. Colors shown after 24 h exposure to indole

Strain	L salts	LB	LB + Fe^{2+}	M9	M9 + Fe^{2+}
P. cepacia G4 PR1	**Deep red/ orange**	Light burgundy	Light burgundy	Light burgundy	Light burgundy
P. cepacia G4	**Light red/ orange**	No color	No color	Dark burgundy	Dark burgundy
P. cepacia G4 induced	**Deep red/ orange**	No color	No color	Light blue	Light blue
P. putida F1	**Dark green/ black**	Green/ brown	Dark green	Green/ black	Green/ black
P. putida F1 induced	**Dark green/ black**	Light brown	Light green	Black	Dark green/black
P. mendocina KR1	No color	No color	No color	**Light blue**	Light blue/yellow
P. mendocina KR1 induced	No color	No color	No color	**Light blue**	Light blue

Fig. 3 Minimal-medium/indole/agar plates indicating the colors generated with various *Pseudomonas* and *E. coli* strains containing TCE-degrading oxygenases. Counterclockwise from top left: *P. putida* F1 (green), *P. cepacia* G4 PR1 (red), *P. mendocina* KR1 (light blue), *P. cepacia* G4 (red), and JM109/pDTG601 (blue)

did serve to corroborate the results of those LB and M9 plates that lacked additional Fe^{2+}. Excess iron was added in an attempt to help increase the enzymatic activity of these iron-containing proteins (Murdock et al. 1993).

To determine whether the pseudomonad oxygenases were responsible for the color formation on the agar plates, *E. coli* cultures harboring the key oxygenases were streaked on LB, LB + indole, M9, and M9 + indole plates (L-salts plates were not used since the *E. coli* strains grow poorly on this medium) with the results shown in Table 2. The *E. coli* host JM109 had no color on either the complex or minimal plates with indole. However, JM109/pDTG601 containing the *todC1C2BA* locus turned blue on M9 + indole plates and dark blue on LB + indole plates. Similarly, JM109/pMY401 with *tmoABCDE* was light blue on M9 + indole plates and black on LB + indole plates. Hence, it is the expression of the oxygenase genes (producing TDO and T4MO) that causes the color development on these indicator plates. Although *E. coli* contains tryptophanase (Murdock et al. 1993) for converting tryptophan into indole (which may be subsequently oxidised to indigo by the oxygenases), the color was more pronounced on the plates containing indole for both recombinants.

To determine whether TOM is responsible for the deep red/orange color seen on the L-salts plates with indole for *P. cepacia* G4 and *P. cepacia* G4 PR1, 1 mM indole was added to overnight *P. cepacia* G4 cultures grown on either glucose or phenol as the sole carbon source. After a 24-h incubation with indole, the phenol-grown culture produced a blue hue and dark precipitate whereas the glucose-grown culture was colorless. Since only the phenol-grown culture produces TOM (glucose-grown *P. cepacia* G4 does not degrade TCE; Sun and Wood 1995), TOM is responsible for the colors seen with indole.

Specific growth rates

The specific growth rate of *P. cepacia* G4 PR1 in M9 glucose minimal medium supplemented with 50 μg/ml kanamycin was 0.80 ± 0.03/h. In MCl medium, the growth rate was reduced slightly (7%) to 0.74 ± 0.06/h. To make certain that exponential growth was monitored, the starting absorbance was kept between 0.001 and 0.06 and absorbance readings from 0.03 to 1.1 were used for exponential growth (this yielded 2–4 h of initial growth, which was ignored, such that an exponential range with four to six absorbance values was used for each of the replicates).

Table 2 Comparison of colors of indole agar plates with recombinant *E. coli* strains containing oxygenases. Colors shown after 24 h incubation with indole. Plates contained 50 μg/ml ampicillin for JM109/pDTG601 and JM109/pMY401

Strain	LB + indole	LB	M9 + + indole	M9
JM109/pDTG601 (with *todC1C2BA*)	Dark blue	Light green	Blue	No color
JM109/pMY401 (with *tmoABCDE*)	Purple/black	Light gray	Light blue	No color
JM109	No color	No color	No color	No color

Discussion

Minimal-indole indicator plates were developed to identify or confirm the presence of the TCE-oxidizing enzymes TDO, T4MO, and TOM on the basis of the different colors produced (green/black, light blue, and deep red/orange respectively). In the presence of other carbon sources (glucose or yeast extract), indole acts as an inducer of the oxygenase genes in these strains since color develops for *P. putida* F1, *P. mendocina* KR1, and *P. cepacia* G4, on M9/glucose or LB plates. Furthermore, the *todC1C2*BA and *tmoABCDE* loci of JM109/pDTG601 and JM109/pMY401 are leaky in *E. coli* since indigo is produced in overnight LB cultures without the addition of the inducer isopropyl-thiogalactoside for the promoters of these cloned oxygenase genes (neither vector has tight control since $lacI^q$ is not present on the plasmids).

Since indole induces expression of the oxygenases in these *Pseudomonas* strains, it was necessary to use *E. coli* strains to verify that the colors stem from the expression of the oxygenase enzymes. Only those *E. coli* cells that harbored the oxygenase genes (*tmoABCDE* or *todC1C2*BA) developed color on indole agar plates; therefore, T4MO and TDO are responsible for the colors observed. Similar results for T4MO were observed by Yen, who detected indigo production in liquid media for *E. coli* strains containing an intact T4MO gene cluster (Yen et al. 1991). In this work, induced and non-induced liquid cultures of *P. cepacia* G4 were also used to show that the indole-related color arises from expression of the TOM genes.

The chloride-free medium that has been developed allows TCE mineralization to be monitored *in situ*. In this novel medium, the constitutive TCE-degrading strain *P. cepacia* G4 PR1 grows well as it degrades and mineralizes TCE to the same extent as in M9/glucose minimal medium. To confirm the presence of *P. cepacia* G4 PR1 in the biofilter, minimal indole plates have been developed which turn deep red/orange in the presence of cells with active TOM. These plates may also be used to distinguish between TOM, TDO, and T4MO.

Acknowledgements This work was supported by the U.S. Army Research Office through an Army Young Investigator Award (for T.K.W.) by the National Science Foundation (REU grant BCS 9210619), and by the California EPA Department of Toxic Substances Control (grant 93-T0527). We are also grateful for the gift of *P. cepacia* G4 and *P. cepacia* G4 PR1 provided by Dr. M.J. Reagin and Prof. M.S. Shields, for the gift of *P. putida* F1 and JM109/pDTG601 by Dr. D.T. Gibson, the gift of *P. mendocina* KR1 and JM83/pMY401 by Dr. K.-M. Yen, for the many valuable discussions with Dr. S. Thomas, and for the helpful comments of Dr. D. Jahng.

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