

## Selection of a *Pseudomonas cepacia* Strain Constitutive for the Degradation of Trichloroethylene†

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**Tn5 insertion mutants of *Pseudomonas cepacia* G4 that were unable to degrade trichloroethylene (TCE), toluene, or phenol or to transform *m*-trifluoromethyl phenol (TFMP) to 7,7,7-trifluoro-2-hydroxy-6-oxo-2,4-heptadienoic acid (TFHA) were produced. Spontaneous reversion to growth on phenol or toluene as the sole source of carbon was observed in one mutant strain, G4 5223, at a frequency of  $\sim 1 \times 10^{-4}$  per generation. One such revertant, G4 5223-PR1, metabolized TFMP to TFHA and degraded TCE. Unlike wild-type G4, G4 5223-PR1 constitutively metabolized both TFMP and TCE without aromatic induction. G4 5223-PR1 also degraded *cis*-1,2-dichloroethylene, *trans*-1,2-dichloroethylene, and 1,1-dichloroethylene and oxidized naphthalene to alpha naphthol constitutively. G4 5223-PR1 exhibited a slight retardation in growth rate at TCE concentrations of  $\geq 530 \mu\text{M}$ , whereas G4 (which was unable to metabolize TCE under the same noninducing growth conditions) remained unaffected. The constitutive degradative phenotype of G4 5223-PR1 was completely stable through 100 generations of nonselective growth.**

Groundwater contamination by organic pollutants is a subject of concern throughout the industrialized world. Chief among these pollutants are those categorized as volatile organics. The largest category of these is the chloroaliphatics, which include the chloroethenes trichloroethylene (TCE), tetrachloroethylene, *trans*-1,2-dichloroethylene (DCE), 1,1-DCE, and vinyl chloride, ranked 1st, 2nd, 3rd, 5th, and less than 10th, respectively, of all volatile organics detected as groundwater contaminants in the United States (31). Nevertheless, their industrial use continues, largely because of their excellent degreasing properties (23, 40). The capability of biologically degrading such contaminants to their basic components (i.e., CO<sub>2</sub> and HCl) at the site of pollution would be of considerable treatment value and would augment current "pump-and-treat" technologies that have been less than completely effective in the remediation of these contaminated environments and offer no permanent solution for disposition of these pollutants short of incineration (37).

Despite the obvious recalcitrance of TCE in the environment, there have been numerous reports of bacteria capable of metabolizing TCE and related isomers. All reports of TCE transformation by anaerobic bacteria indicate a very slow process (3-5, 14, 19) which is often associated with the production of vinyl chloride (41). The metabolism of TCE by aerobic bacteria, although more rapid than that by anaerobic bacteria, is not observed in environments without aromatic or aliphatic copollutants because TCE is merely a cooxidative substrate for the requisite oxygenases and not an inducer of these enzymes. All TCE-degrading bacteria, with the exception of *Nitrosomonas europaea* (2, 39), require the addition of an exogenous inducer substrate such as toluene (18, 26-28, 43, 46), phenol (15, 25, 27), methane (13, 16, 22, 30, 38, 44), isoprene (9), propane (42), or 2,4-dichlorophe-

noxyacetic acid (15) to induce the enzymes required for the degradation of TCE.

A Tn5-induced mutant of *Pseudomonas cepacia* G4 (Tom<sup>-</sup>) that does not express toluene *ortho*-monooxygenase (TOM) (35) but spontaneously reverts to the constitutive expression of TOM is described. This revertant no longer requires aromatic induction of the TOM pathway enzyme(s) in order to degrade TCE.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. A medium consisting of yeast extract (0.05%), glucose (0.1%), and basal salts medium (BSM [34]) (YGM) (pH 7.0) was used to grow cells for the determination of *m*-trifluoromethyl phenol (TFMP) degradation rates. Other culture conditions for *P. cepacia* and its derivatives have been described elsewhere (35). *Escherichia coli* strains were grown on Luria-Bertani medium (24) with the appropriate selective antibiotics.

**Tn5 mutagenesis.** The introduction and subsequent transposition of Tn5 into the genome of *P. cepacia* G4 was carried out via a triparental mating between G4, *E. coli* C600(pRZ102), and *E. coli* HB101(pRK2013). Overnight cultures of each of the parents were diluted twofold with fresh Luria-Bertani medium and grown for an additional 2 h at 30°C. One milliliter of each of the three cultures was combined in a single 3-ml syringe, and the cells were transferred to a 0.45- $\mu\text{m}$ -pore-size nitrocellulose filter, which was transferred (cell side up) to a fresh Luria-Bertani agar plate and incubated overnight at 30°C. The following day, the cells were suspended by vortexing the filters with 3 ml of BSM (34). Tn5-induced mutants of *P. cepacia* G4 were selectively grown on BSM-purified agar containing 20 mM sodium lactate and 50  $\mu\text{g}$  of kanamycin sulfate per ml.

**Detection of Tom<sup>-</sup> *P. cepacia* mutants.** Mutants defective in the toluene-degradative pathway were detected by their inability to oxidize TFMP (Aldrich Chemical Co., Milwau-

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TABLE 1. Bacterial strains

Strain	Description <sup>a</sup>	Reference or source
<i>P. cepacia</i>		
G4	Wild type (Phe <sup>+</sup> TCE <sup>+</sup> )	35
G4-5220	G4::Tn5 (Phe <sup>-</sup> TCE <sup>-</sup> ); <i>o</i> -xylene revertible <sup>b</sup>	This study
G4-5223	G4::Tn5 (Phe <sup>-</sup> TCE <sup>-</sup> ); phenol revertible <sup>b</sup>	This study
G4-5227	G4::Tn5 (Phe <sup>-</sup> TCE <sup>-</sup> ); <i>o</i> -xylene revertible <sup>b</sup>	This study
G4-5231	G4::Tn5 (Phe <sup>-</sup> TCE <sup>-</sup> ); phenol revertible <sup>b</sup>	This study
G4-5223-PR1	Tom <sup>c</sup> phenol revertant of G4-5223 (TCE <sup>+/-</sup> )	This study
<i>E. coli</i>		
C600(pRZ102)	pColE1::Tn5 ( <i>mob</i> <sup>+</sup> Km <sup>r</sup> ); suicide vector for delivery of Tn5 into <i>Pseudomonas</i> species	17
HB101(pRK2013)	pColE1::RK2 Tra <sup>+</sup> Km <sup>r</sup> ; mobilization vector for pRZ102	10

<sup>a</sup> Km<sup>r</sup>, resistant to 50 µg of kanamycin sulfate per ml; Phe<sup>+</sup> and Phe<sup>-</sup>, ability and inability, respectively, to degrade phenol; TCE<sup>+</sup> and TCE<sup>-</sup>, inducible and noninducible TCE degradation, respectively; TCE<sup>+/-</sup>, constitutive TCE degradation; Tom<sup>c</sup>, constitutive for TOM; Tra<sup>+</sup>, transmissible plasmid.

<sup>b</sup> Revertible indicates that a heavy inoculum of cells to minimal agar containing a specific carbon and energy source gave rise to only a few colonies capable of growth on this medium.

kee, Wis.) to the yellow transformation product 7,7,7-trifluoro-2-hydroxy-6-oxo-2,4-heptadienoic acid (TFHA) (8) as previously described (35). Because of the high water solubility of TFHA, direct incorporation into solid growth medium gave only a diffusive yellow product that could not be associated with a particular colony. In order to circumvent this difficulty, nitrocellulose filters (7-cm diameter, 0.45-µm pore size; Schleicher and Schuell, Keene, N.H.) were saturated with 5 mM TFMP and air dried. Colonies from BSM-lactate agar plates grown in the presence of toluene vapor (34) were lifted onto the surfaces of the TFMP-impregnated filters. Wild-type G4 colonies became yellow within 10 min. Mutant colonies lacking TOM or catechol-2,3-dioxygenase (C230) remained colorless. C230 mutants were distinguished from mutants lacking TOM through brown autoxidation products of 3-methylcatechol accumulated in the presence of phenol or toluene. Since the colonies began desiccation immediately upon transfer to the dry membranes, all associated TFHA production remained localized to individual colonies.

**Enzyme assays and TCE analysis.** C230 and hydroxymuconic semialdehyde dehydrogenase were assayed as previously described (35). TCE analysis was carried out by gas chromatography of pentane extracts of culture medium as previously described (35).

**TCE degradation assays.** TCE degradation was assayed following an overnight incubation of washed cells suspended in 2 ml of BSM containing 20 µM TCE (calculated as though all TCE were in solution) in a 12-ml glass vial, crimp sealed with a Teflon-lined rubber septum. Pentane extraction (2 ml) allowed measurement of residual TCE concentrations by gas chromatography with an electron capture detector.

Rates of TCE degradation were determined with cell

suspensions in a 50-ml glass syringe with a Teflon plunger and no air headspace as previously described (12). This assay allowed multiple nondestructive samplings (1 ml each) without the introduction of an air headspace.

**Growth and TCE metabolism.** TCE was added to 100 ml of YGM and a 1% inoculum of an overnight Luria-Bertani culture of each strain to be tested, in 500-ml sidearm Erlenmeyer flasks, to give four TCE concentrations: 35, 218, 550, and 3,260 µM (as determined by direct extraction of culture liquid). Growth was achieved at 30°C with shaking at 200 rpm. Changes in cell density were monitored with a Klett spectrophotometer. Since strain G4 metabolizes TCE only following exposure to aromatic inducers (25–28, 35), it serves as an excellent negative metabolic control when uninduced. Any toxic effect of TCE on uninduced G4 must necessarily be due to the direct toxicity of TCE to the organism and not to any consequences of its metabolism.

**Temperature, pH, oxygen, and salinity effects.** The effects of temperature, oxygen concentration, pH, and salinity on the rate of TCE degradation by the *P. cepacia* revertant G4-5223-PR1 (hereafter referred to as PR1) were investigated. Various concentrations of dissolved oxygen were established in Tris-HCl-buffered BSM (10 mM Tris-HCl [pH 8.0]) was substituted for the phosphate components of BSM) at 23°C. This was accomplished by sparging autoclaved Tris-HCl BSM with pure O<sub>2</sub> for various time intervals. O<sub>2</sub> concentrations were determined with a Gilson Oxygraph (Gilson Medical Electronics Inc., Middleton, Wis.). Temperature and pH effects in BSM adjusted with HCl or NaOH to the appropriate pH values were determined. Temperatures were controlled by submersion of the reaction vessel (a 50-ml glass syringe with a Teflon plunger) in a circulating water bath. Salinities were adjusted with an artificial seawater medium consisting of the following (grams per liter): NaCl (20 or 8), MgSO<sub>4</sub> · 7H<sub>2</sub>O (1.0), KCl (0.7), NH<sub>4</sub>NO<sub>3</sub> (1.0), and K<sub>2</sub>HPO<sub>4</sub> (2.6). Homogeneous suspensions of cells and reactants were ensured throughout the experiment by the inclusion of a stir flea inside the syringe propelled by a submersible magnetic stirrer. Because this was a sealed system, only initial linear rates of degradation were recorded (usually ≤10 min). Data were collected over 20 min at 2-min intervals. Unless otherwise noted, TCE degradation was carried out in BSM (pH 7.0) with 7.7 mg of O<sub>2</sub> per liter, at 23°C.

## RESULTS

**Tn5-induced Tom<sup>-</sup> mutants.** Putative Tn5-induced mutants of G4 were selected as kanamycin-resistant (50 µg/ml), lactate-utilizing colonies from the triparental mating *E. coli* C600(pRZ102) × *E. coli* HB101(pRK2013) × *P. cepacia* G4. Approximately 2 × 10<sup>5</sup> such colonies were assayed for their inability to metabolize TFMP by pulling them onto TFMP-impregnated nitrocellulose filters. Four Tn5-induced mutants of G4 were isolated by this technique: G4-5220, G4-5223, G4-5227, and G4-5231. All were unable to produce TFHA from TFMP. G4-5220 produced catechol from phenol and other aromatic substrates as indicated by a brown discoloration of the medium (Table 2). The metabolic transformations affected in these mutants are indicated in Fig. 1.

**Isolation of a constitutive TCE degrader.** The four Tn5-induced mutants were challenged for growth on BSM plates containing 2 mM phenol as the sole carbon source. Only G4-5223 and G4-5231 gave rise to revertant colonies. Fifty revertant colonies of each were transferred to BSM-lactate. These colonies were in turn lifted onto 7-cm-diameter nitro-

TABLE 2. Growth of Tn5-induced mutants on single carbon sources

Carbon source	Growth and metabolism of aromatic substrates by the following strains of <i>P. cepacia</i> <sup>a</sup> :					
	G4	G4-5220	G4-5231	G4-5227	G4-5223	PR1
Phenol	+	+ B	R	-	R	+
Toluene	+	- B	R	-	R	+
Benzene	+	- B	R	-	R	+
<i>o</i> -Cresol	+	- B	R	-	R	+
<i>m</i> -Cresol	+	- B	R	-	R	+
<i>p</i> -Cresol	+	- B	R	-	R	+
<i>o</i> -Xylene	+	R <sup>b</sup>	R	R <sup>b</sup>	R	+
<i>m</i> -Xylene	-	-	-	-	-	-
<i>p</i> -Xylene	-	-	-	-	-	-

<sup>a</sup> Growth (+) or nongrowth (-) on BSM-purified agar plates containing each indicated sole carbon source in the vapor phase (toluene, benzene, and xylenes) or at 0.5 mM (cresols) or 2 mM (phenol). The presence of brown discoloration of the medium (B) and revertants selectable from a heavy inoculum that are subsequently transferable on the same carbon source (R) are indicated.

<sup>b</sup> Revertants that subsequently grew with *o*-, *m*-, and *p*-xylene as sole carbon sources but were incapable of growth on any of the other substrates tested.

cellulose discs impregnated with TFMP and compared with wild-type G4 similarly grown on this noninducing medium. All revertant colonies of G4-5223 and G4-5231 were constitutive for yellow-TFHA production. The frequency of reversion of G4-5223 to utilization of phenol was subsequently measured at a frequency of  $\sim 1 \times 10^{-4}$  per generation. The reversion rates of other revertible Tn5-induced mutants were not measured. One such revertant of G4-5223, G4-5223-PR1, was selected for further characterization.

TOM activity cannot be detected in cell-free preparations but can be inferred from intact cells by measurement of the catabolism of TFMP to TFHA (35). The rate of the production of TFHA from TFMP is therefore a corollary of its potential rate of toluene, and therefore TCE, degradation. Samples of G4 taken during growth on YGM containing 2 mM phenol (as an inducer of TOM activity) were assayed for their rates of TFHA production from TFMP, and the rates were plotted and compared with that of PR1 grown on YGM without inducer (Fig. 2). PR1 can be seen to catabolize TFMP with an essentially unchanged rate throughout its growth curve despite the absence of TOM inducers. G4 is capable of attaining nearly the same rate of TFHA production, but only late in the growth cycle, and this maximal rate is maintained for only a short time. The loss of activity by G4 is presumably due to utilization of the inducer (phenol).

G4 grown on lactate without inducer was washed and exposed to 56  $\mu$ M TCE. Uninduced, it was unable to affect the TCE concentration over a 19-h period. PR1, prepared in the same manner, removed all detectable TCE during the same time period (Fig. 3).

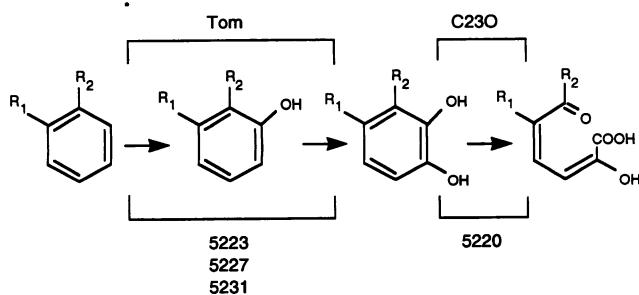


FIG. 1. Pathway of aromatic catabolism by *P. cepacia* G4. The two enzyme activities associated with this pathway and the respective Tn5 mutants affecting them are indicated. R<sub>1</sub>, hydrogen or second methyl group; R<sub>2</sub>, hydrogen or methyl group.

**C23O constitutivity.** The first two enzymes recognized as part of the toluene-degradative pathway of G4 are TOM and C23O (35). Only C23O can be assayed in cell lysates. Since both of these activities are required for the conversion of TFMP to TFHA (the reaction used to select PR1), it was of interest to establish to what degree C23O had become constitutive in PR1. G4, G4-5223, and PR1 were assayed for C23O activity against methylated and nonmethylated substrates, with and without phenol induction (Table 3). PR1 (unlike G4 or G4-5223) constitutively produces C23O at levels comparable to those produced by G4 in the presence of phenol as an inducer.

**Growth and TCE metabolism.** The growth rates of PR1 and G4 in YGM were monitored in the presence of TCE at concentrations of 35, 210, 530, and 3,260  $\mu$ M. There was no observed effect on the growth rate of either strain at 35 and 210  $\mu$ M TCE, but a substantial depression of growth rate was evident in both strains at 3,260  $\mu$ M. At a liquid phase TCE concentration of 530  $\mu$ M, the growth of PR1 was noticeably depressed whereas that of G4 remained unaffected (Fig. 4). This depression of PR1 growth rate cannot be attributed to the toxicity of unaltered TCE and must therefore be a direct consequence of TCE metabolism.

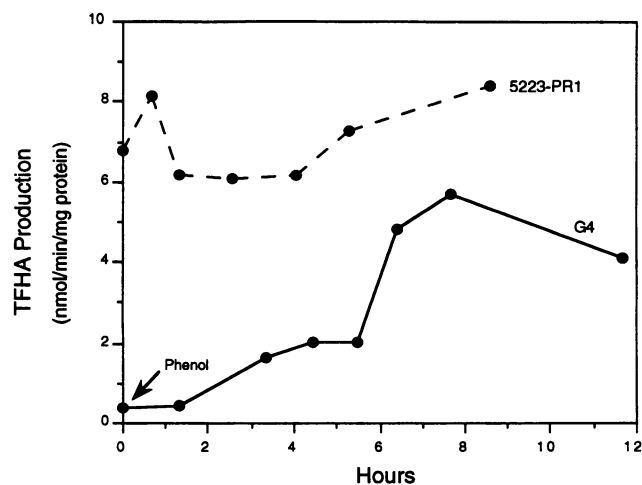


FIG. 2. Rates of TFHA production from TFMP during batch growth of *P. cepacia* G4 and PR1. Phenol (2 mM) was added at time zero to the G4 culture only (as indicated by the arrow).

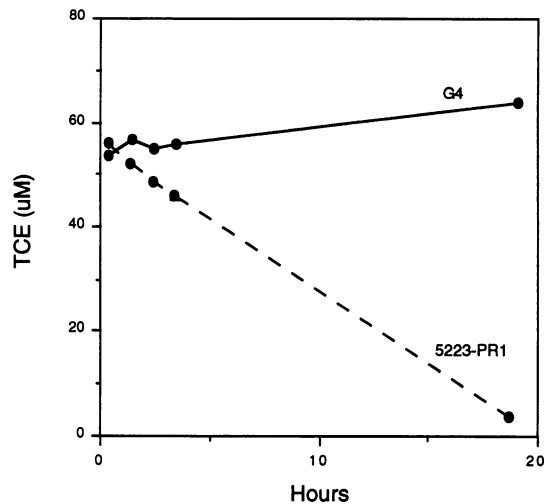


FIG. 3. TCE degradation by washed cells grown in BSM-lactate.

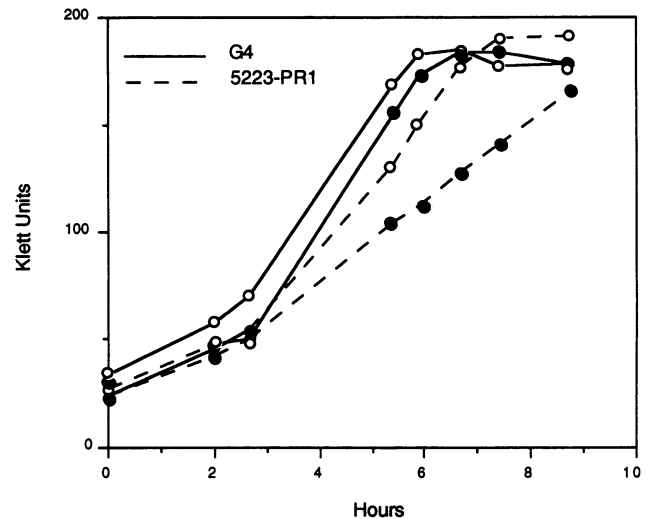


FIG. 4. Growth effects of TCE metabolism. Determination of the growth curves of the indicated strains was conducted at a TCE aqueous-phase concentration of 530  $\mu\text{M}$ .

#### Effect of physical variables on the TCE degradation rate.

The effects of temperature, pH, oxygen, and salinity on the rate of TCE degradation by PR1 were measured to determine the probable impact of such environmental variables during in situ aquifer treatments. The most profound effect on the rate of TCE degradation was found at the lowest temperatures used (Fig. 5a), at which, despite cooling to 4°C, PR1 maintained approximately 30% of the rate of TCE degradation measured at 30°C. TCE degradation rates of approximately 20 and 45% (relative to the maximal value at pH 7) were found at pH extremes of 4 and 9, respectively (Fig. 5b). Little effect on TCE degradation rates was observed over the ranges of salinity (0 to 20‰) (Fig. 5c) or oxygen (2.8 to 31.3 mg/liter) (Fig. 5d) tested. Uninduced G4 is included in each experiment as a metabolic control group since it has a cell physiology identical to that of PR1 but cannot oxidize TCE.

**Genetic stability.** Batch cultures of PR1 were transferred with sequential dilutions to yield a total of 100 generations in two media, BSM-lactate and BSM-lactate containing kanamycin sulfate (50  $\mu\text{g/ml}$ ), to independently determine the stability of the Tn5 element as well as the constitutive phenotype. The resulting cell populations were each tested for kanamycin resistance and the ability to constitutively transform TFMP to TFHA. A total of 1,394 colonies (i.e., 444 from the lactate-kanamycin plates and 950 from the lactate plates) were found to retain their ability to constitutively oxidize TFMP.

TABLE 3. C23O activity

Strain	Inducer	C23O activity with the following assay substrates (nmol/min/mg of protein) <sup>a</sup> :	
		Cat	3mCat
G4	None	2.1	3.7
G4	Phenol	53.6	62.5
G4-5223	None	0.07	2.2
G4-5223	Phenol	13.1	31.4
G4-5223-PR1	None	156.0	50.4
G4-5223-PR1	Phenol	48.0	34.9

<sup>a</sup> Cat, catechol; 3mCat, 3-methylcatechol.

**Degradation of chlorinated ethylenes other than TCE.** The ability of PR1 to degrade chlorinated ethylenes in addition to TCE was tested. TCE, 1,1-DCE, *cis*-1,2-DCE, *trans*-1,2-DCE, and tetrachloroethylene (initial concentrations of ca. 10  $\mu\text{M}$ , as though all were in aqueous solution) were tested in batch TCE degradation assays with an air headspace. The proportion of these compounds remaining after an overnight incubation is given in Table 4. Uninduced G4 was included as a metabolic control and clearly fails to demonstrate any detectable background constitutive degradation of any compound tested. All chloroaliphatics tested, with the exception of tetrachloroethylene, were degraded by PR1 without added inducer. No attempt was made to optimize the cell concentration or incubation parameters for maximal degradation of the substrates. An unidentified compound was detected in

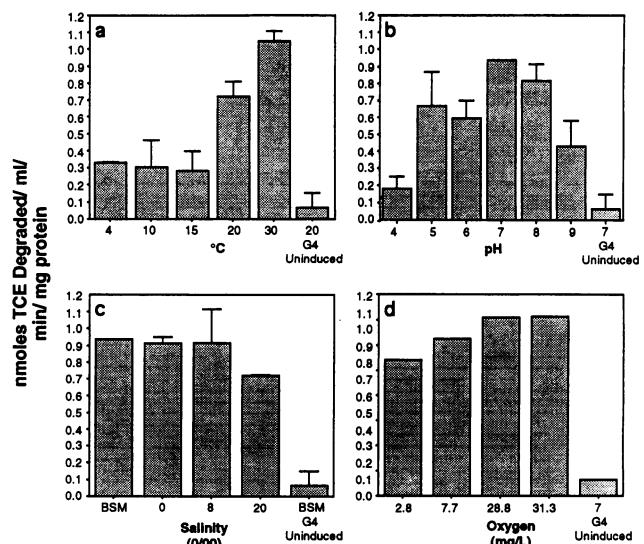


FIG. 5. TCE degradation rates for PR1 for various ranges of environmental conditions. Initial TCE concentration,  $\sim 6 \mu\text{M}$ .

TABLE 4. Degradation of chloroaliphatics

Strain	% remaining <sup>a</sup>				
	1,1-DCE	<i>cis</i> -1,2-DCE	<i>trans</i> -1,2-DCE	TCE	PCE
Control (uninoculated)	100 ± 2	100 ± 4	100 ± 9	100 ± 3	100 ± 7
G4 (uninduced)	104 ± 3	ND	107 ± 5	133 ± 5	103 ± 7
G4-5223-PR1	50 ± 3	12 ± 9 <sup>b</sup>	0 <sup>b</sup>	2 ± 2	104 ± 3

<sup>a</sup> Amounts of substrates (starting concentration, ca. 10 μM) remaining, expressed as percentages of the uninoculated controls ± standard deviations. PCE, perchloroethylene. ND, not determined.

<sup>b</sup> An unidentified product was detected in this sample by gas chromatography-electron capture detector.

culture extracts of PR1 containing either *cis*- or *trans*-1,2-DCE. Each was detected as an electron capture detector peak during gas chromatography. Each compound demonstrated considerably longer retention times than either parent compound, and each is presumed to be a metabolite.

### DISCUSSION

Aerobic bacterial metabolism of TCE results from the metabolic action of enzymes not induced by TCE (2, 9, 16, 25–27, 42, 44, 45, 47, 48). In the present work, a method for the generation of a nonrecombinant *P. cepacia* strain constitutive for the oxidation of TCE was presented. Three toluene oxygenases have been cloned in *E. coli* under alternate promoter control and thus no longer require aromatic induction (18, 46, 48). This is the first report of a nonrecombinant bacterium, derived from a toluene-inducible, TCE cometabolizing parent, that constitutively degrades TCE.

One Tom<sup>-</sup> *P. cepacia* mutant, G4-5223, reverted to phenol and toluene utilization at a very high frequency (~1 × 10<sup>-4</sup> per generation). One such revertant, PR1, was found to have maintained the Tn5-encoded kanamycin resistance and reverted to phenol and toluene utilization through a circumvention of the normally stringent regulatory mechanism, inducible by aromatics such as toluene or phenol in G4. Expression of the first two assayable enzymic activities of its assimilatory pathway for these compounds, TOM and C23O, was found to be constitutive in PR1. As a result, TCE was degraded without exposure to aromatic inducers. The rate of conversion of TFMP to TFHA (a reaction requiring both TOM and C23O) attained by this constitutive derivative was approximately 8 nmol of TFHA per min per mg of protein. This was comparable to the rate of 6.5 nmol of TFHA per min per mg of protein attained by G4 grown under similar batch culture conditions, with 2 mM phenol as an inducer. The maximal rate of TCE degradation measured for PR1 produced in batch culture was ~1 nmol/min/mg of protein, which is comparable to that reported for chemostat-grown, phenol-induced G4 (11).

Growth and metabolism profiles for the four Tn5-induced mutants of the TOM pathway reported indicate at least three distinct phenotypes (i.e., one nonrevertible [with phenol selection] Tom<sup>-</sup> mutant, two phenol-revertible Tom<sup>-</sup> mutants, and one C23O mutant not revertible with phenol selection). All mutants (Tom<sup>-</sup> or C23O<sup>-</sup>) gave rise to revertants capable of using *o*-xylene as a carbon source. This is particularly interesting in the cases of G4-5220 (C23O<sup>-</sup>) and G4-5227 (Tom<sup>-</sup>) in that they were exclusively revertible with *o*-xylene and not any other substrate used. Revertants of G4-5223 and G4-5231 to growth on *o*-xylene were also able to grow on toluene, phenol, benzene, and *o*-cresol, whereas *o*-xylene-grown revertants of G4-5220 and G4-5227 remained

unable to grow with toluene, phenol, benzene, and *o*-cresol. The nature of these reversion patterns is the subject of current investigations, but the preliminary interpretation is that G4 contains an alternate pathway for the catabolism of xylene that is not readily recruited when the TOM pathway is functional. The growth of G4-5220 on phenol was not due to reversion, because previous C23O mutants were found to be capable of phenol growth as well (presumably because of a catechol *ortho* fission capability) and because the accumulation of catechol was still obvious from the brown discoloration of the medium. All phenol-utilizing revertants from G4-5223 and G4-5231 tested were found to be constitutive for the conversion of TFMP to TFHA.

C23O was found to be constitutively expressed in PR1 but still inducible in G4-5223. It is unlikely that a partial block in the transcription of a gene segment that encodes both TOM and C23O is responsible. Our failure to detect either TCE metabolism or TFMP hydrolysis by phenol-induced G4-5223 indicates that the Tn5 insertion caused a complete cessation of Tom expression. One possible explanation for this is that the C23O gene is part of the same polycistronic message that encodes TOM but responds to a second promoter specific to the C23O gene (as indicated by the additional expression of C23O in G4-5223 in the presence of phenol).

Evidence has accumulated for all bacterial oxygenases studied that are capable of TCE metabolism indicating that such oxidative metabolism produces highly reactive products which are toxic to the bacterium. Significant damage to bacteria through intracellular reactions between the reactive TCE product and cell components has been reported elsewhere for methanotrophs (1, 16, 29), *Pseudomonas putida* F1 (44), and *N. europaea* (32). The effects of TCE oxidation have been shown to be quite mild for the isoprene-oxidizing bacteria *Alcaligenes denitrificans* subsp. *xylooxidans* JE 75 and *Rhodococcus erythropolis* JE 77 (9). Wild-type G4 and its constitutive derivative, PR1, provide an ideal system to determine whether TCE metabolism affects *P. cepacia*, since uninduced G4 does not affect TCE. A slight depression in the growth rate of PR1 was seen at a measured liquid phase TCE concentration of 554 μM, but this was not the case for G4. This was a TCE concentration similar to that reported by Ewers et al. (ca. 2 mM, as though all TCE was in solution) (9) at which a depression in growth rate in *A. denitrificans* and *R. erythropolis* was noticeable.

PR1 appears to be capable of the complete metabolism of *cis*- and *trans*-DCE and TCE, whereas perchloroethylene remains unaffected. 1,1-DCE, however, was only partially metabolized (~50%) in these studies (Table 4). This observation is analogous to that of Ewers et al. (9), in which the metabolism of 1,1-DCE by isoprene-induced bacteria was only a fraction of the TCE metabolized. In the case of the isoprene oxidizers, the extreme instability of the 1,1-DCE epoxide (half life, 2 s) (21) and its resulting potential cyto-

toxicity were cited as probable mechanisms of inactivation. This appears to be a plausible explanation for our observation with PR1 as well. The genetic alteration leading to constitutivity in PR1 was completely stable over 100 generations of nonselective growth and during strain maintenance. The genetic nature of this alteration is a subject of current investigations. Especially relevant may be the high rate at which the constitutive colonies arise from the mutant G4-5223 ( $1 \times 10^{-4}$  per generation). Many possible explanations exist. Tn5 has been reported to provide an outwardly constitutive transcriptional promoter in *P. putida* (7) and *Alcaligenes eutrophus* (6). However, this possibility does not explain why the initial Tn5 insertion mutant, G4-5223, had a complete loss of activity and why only the derivative strain, PR1, was constitutive. A second possibility is that of constitutive promoter-bearing insertion sequences native to *P. cepacia*. More than 13 such transcriptional activating insertion elements within *P. cepacia* with very high rates of transposition have been described elsewhere (20, 33, 36). Other possibilities include indirect suppression of the original Tn5 insertion mutant through IS50 or Tn5 transposition to other sites or spontaneous up-mutations in the expression of one or more affected genes. The precise genetic nature of the revertible phenotype described for G4-5223 in this paper remains undetermined.

The ability of PR1 to degrade TCE under a range of physical conditions expected for TCE-contaminated aquifers is encouraging for its consideration as an in situ biodegradative agent.

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