Effect of Trichloroethylene on the Competitive Behavior of Toluene-Degrading Bacteria

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The influence of trichloroethylene (TCE) on a mixed culture of four different toluene-degrading bacterial strains (*Pseudomonas putida* mt-2, *P. putida* F1, *P. putida* GJ31, and *Burkholderia cepacia* G4) was studied with a fed-batch culture. The strains were competing for toluene, which was added at a very low rate (31 nmol mg of cells [dry weight]$^{-1}$ h^{-1}). All four strains were maintained in the mixed culture at comparable numbers when TCE was absent. After the start of the addition of TCE, the viabilities of *B. cepacia* G4 and *P. putida* F1 and GJ31 decreased 50- to 1,000-fold in 1 month. These bacteria can degrade TCE, although at considerably different rates. *P. putida* mt-2, which did not degrade TCE, became the dominant organism. Kinetic analysis showed that the presence of TCE caused up to a ninefold reduction in the affinity for toluene of the three disappearing strains, indicating that inhibition of toluene degradation by TCE occurred. While *P. putida* mt-2 took over the culture, mutants of this strain which could no longer grow on *p*-xylene arose. Most of them had less or no *meta*-cleavage activity and were able to grow on toluene with a higher growth rate. The results indicate that cometabolic degradation of TCE has a negative effect on the maintenance and competitive behavior of toluene-utilizing organisms that transform TCE.

A limited number of chlorinated aliphatic hydrocarbons can support bacterial growth by serving as a source of carbon and energy (17). In these cases, treatment of polluted sites or waste streams can be performed by using systems in which the number of desirable microorganisms increases because they proliferate at the expense of the contaminants. However, many chlorinated compounds are biodegradable only by cometabolic conversion. In this case, the xenobiotic compound does not cause a selective increase in the population of the active microorganisms. A primary substrate must be present for growth and maintenance, which, however, does not necessarily select for the desired degradative activity. Besides, conversion of the cosubstrate could lead to toxic products which inhibit the most active organisms.

The cometabolic conversion of trichloroethylene (TCE) by nonspecific oxygenases of aerobic bacteria is an example of a process which may harm the bacteria that execute the degradation reaction. The reaction uses reducing equivalents (9), and TCE can be a competitive inhibitor of the conversion of the primary growth substrate, which is usually also required to induce the oxidative enzyme. Furthermore, TCE conversion can be accompanied by toxic effects; i.e., cell damage may occur due to nonspecific reactions of TCE conversion products with cell components, as was shown with cultures of *Pseudomonas putida* F1 and *Methylosinus trichosporium* OB3b (27, 35). These toxic effects can lead to a large increase in maintenance energy demand, as was observed in a nongrowing, toluene-limited, fed-batch culture of *Burkholderia cepacia* G4, which converted TCE during cultivation on toluene as the primary substrate (22). However, despite these negative effects, cometabolic conversion is the only possibility for aerobic biodegradation of TCE and deserves significant attention,

since no organisms which can grow on this compound are known.

When organisms are applied for cometabolic TCE degradation during in situ bioremediation processes, they might frequently face situations with very low substrate concentrations. Also, in reactors employing biofilms, the amount of growth substrate that is added is usually kept as low as possible to prevent the formation of excess biomass. However, under energy-limiting conditions, negative effects of TCE conversion, such as an increased maintenance coefficient, could give TCE degraders a large disadvantage compared to organisms that also use the primary substrate but do not degrade TCE. This could limit the long-term stability of a treatment process and lead to reduced conversion rates.

The purpose of the work described in this paper was to determine the competitiveness of TCE-degrading bacteria at very low primary substrate concentrations in the absence and presence of TCE. Toluene was taken as a model primary substrate.

Toluene degradation can start with oxidation of the methyl group or by direct oxidation of the aromatic ring. The first route is used by *P. putida* mt-2, which converts toluene via benzylalcohol, benzaldehyde, and benzoate to catechol (1). Direct oxidation of the aromatic ring is performed by, for example, *B. cepacia* G4 (25, 26) and *P. putida* F1 (34). *B. cepacia* G4 converts toluene via *o*-cresol to 3-methylcatechol (3MC) by two subsequent monooxygenase steps (32). *P. putida* F1 uses a dioxygenase to convert toluene to toluene dihydrodiol, which is subsequently oxidized to 3MC (34).

To study the effect of TCE on the competition for small amounts of toluene between microorganisms, four different toluene degraders were cultivated together in a fed-batch culture, which allows the use of very low toluene concentrations (5). Two of the strains (*B. cepacia* G4 and *P. putida* F1) are well known for their ability to cometabolically convert TCE when toluene is the primary substrate. *P. putida* mt-2 is unable to degrade TCE, while *P. putida* GJ31 (23) converts TCE slightly.

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TABLE 1. Substrate supply parameters

Parameter (unit)	Value in fed-batch culture grown on:	
	Toluene (0–40 days)	Toluene + TCE (40–83 days)
Airflow (ml min^{-1})	53.2	31.8
Toluene flow (ml min^{-1})	2.0	2.0
TCE flow (ml min^{-1})		22.5
[Toluene] in ingoing gas stream (μM)[a]	13.3	13.0
[TCE] in ingoing gas stream (μM)[a]		7.1

[a] Average taken from gas chromatography measurements.

The results show that when TCE was added to the culture, *P. putida* mt-2 and mutants thereof became the dominant organisms, which caused a loss of the TCE degradation capacity.

MATERIALS AND METHODS

Nomenclature. The following parameters are used in this paper: a_d, specific decay rate (minute^{-1}); C, substrate concentration (micromolar); H, dimensionless Henry's coefficient; k_La, mass transfer rate coefficient (minute^{-1}); K_i, inhibition constant (micromolar); K_m, Michaelis-Menten constant (micromolar); K_s, Monod constant (micromolar); $\mu_{\max}$, maximal specific growth rate (minute^{-1}); $r_{\max}$, maximal specific substrate conversion rate (micromoles milligram of cells [dry weight]$^{-1}$ minute^{-1}); t, time (minutes); V, volume (liters); X, concentration of biomass (milligrams of cells [dry weight] liter^{-1}); and Y, growth yield (milligrams of cells [dry weight] micromole^{-1}). Subscripts denote the following parameters: g, gas phase; l, liquid phase; and 0, time zero.

Bacterial strains. *B. cepacia* G4 (26) was a gift from M. S. Shields (U.S. Environmental Protection Agency, Gulf Breeze, Fla.). *P. putida* F1 (34) was provided by L. P. Newman (University of Minnesota, St. Paul). *P. putida* GJ31 was previously characterized (23), and *P. putida* mt-2 (1) was a gift from W. Duetz (RIVM, Bilthoven, The Netherlands).

Culture conditions. The organisms were grown in a fed-batch fermentor with a working volume of 2.5 liters. The mineral medium (MMV) contained (per liter) 5.3 g of Na$_2$HPO$_4$ · 12H$_2$O, 1.4 g of KH$_2$PO$_4$, 0.2 g of MgSO$_4$ · 7H$_2$O, 1.0 g of (NH$_4$)$_2$SO$_4$, 1 ml of a vitamin solution (16), and 5 ml of a trace element solution. The trace element solution contained (per liter) 780 mg of Ca(NO$_3$)$_2$ · 4H$_2$O, 200 mg of FeSO$_4$ · 7H$_2$O, 10 mg of ZnSO$_4$ · 7H$_2$O, 10 mg of H$_3$BO$_3$, 10 mg of CoCl$_2$ · 6H$_2$O, 10 mg of CuSO$_4$ · 5H$_2$O, 4 mg of MnSO$_4$ · H$_2$O, 3 mg of Na$_2$MoO$_4$ · 2H$_2$O, 2 mg of NiCl$_2$ · 6H$_2$O, and 2 mg of Na$_2$WO$_4$ · 2H$_2$O. Each component of the medium was autoclaved separately, except the phosphates and the trace elements, which were autoclaved together, and the vitamin solution, which was filter sterilized prior to addition. The pH was adjusted to 7.2 with autoclaved 0.5 M NaOH and 0.25 M H$_2$SO$_4$. The temperature was set at 28°C, and the impeller speed was set at 1,500 rpm.

Toluene was supplied to the culture via the gas phase. This was done by leading an airflow via a glass filter (P3; Elgebe, Leek, The Netherlands) through ice-cold toluene prior to addition to the culture. TCE was added via the gas phase from a gas cylinder containing 475 ppm of TCE in air (AGA Gas BV, Amsterdam, The Netherlands). Extra water-saturated air was added to the culture to supply sufficient oxygen. The flow rates of the gases are given in Table 1. All gas flows were filter sterilized before addition to the culture. Flows were controlled with mass flow controllers (type F201C-FA-11-V; 0 to 5 ml min^{-1}, 0 to 20 ml min^{-1}, and 0 to 500 ml min^{-1}; Bronkhorst High-Tec B.V., Veenendaal, The Netherlands). The outgoing gas stream was led through a water column under slight overpressure, which facilitated the detection of possible leakage.

Analysis of the culture. The culture density of each sample was estimated by measuring the optical density at 450 nm (OD$_{450}$) on a Hitachi 100-60 spectrophotometer and by determining the protein concentration as described by Lowry et al. (21) with bovine serum albumin as a standard. The dry weight of the culture was determined after 40 days by centrifuging duplicate 100-ml samples of culture (15 min, 6,000 × g, 4°C), washing the pellets with the same volume of cold demineralized water, and drying the pellets to a constant weight in a preweighed aluminum cup for 2 to 3 days at 80°C.

The viability of the culture was determined by diluting a culture sample and counting the number of colonies (CFU) after the sample was plated on 1.5% agar plates containing either rich medium (nutrient broth [NB] plates) or mineral medium to which 10 mg of yeast extract liter^{-1} was added (MMY) instead of the vitamin solution. One day after plating, 5 to 10 μl of pure toluene (Tol plates) was added on a paper filter disk in the cover of the petri dish. This was done to prevent the immediate exposure of the cells to high toluene concentrations. The plates were incubated at 30°C. Colonies on rich plates appeared after 1 to 2 days, and those on Tol plates were visible after 3 to 4 days.

The different strains in the mixed fed-batch culture were distinguished on NB plates by colony morphology and growth rate and by plating diluted culture

samples on selective plates followed by counting the CFU on each plate. The selective plates were made by adding a particular volatile carbon source on a paper filter disk which was placed in the cover of MMY plates 1 day after plating. The plates were incubated at 30°C. Colonies appeared after 3 to 4 days. Of the four strains studied, only *P. putida* GJ31 is able to grow on chlorobenzene plates. *P. putida* mt-2 could be identified by growth on *p*-xylene plates, and *B. cepacia* G4 could be identified by growth on *o*-cresol plates. The number of *P. putida* F1 cells was determined by the difference between the viability on *m*-cresol plates (on which both *P. putida* F1 and *B. cepacia* G4 grow) and the viability on *o*-cresol plates (on which *P. putida* F1 does not grow). Also, *P. putida* F1 and *B. cepacia* G4 could be distinguished on *m*-cresol plates because the colonies of *B. cepacia* G4 were larger than the colonies of *P. putida* F1.

The percentage of cells of each strain that formed colonies on NB plates and were also able to grow on toluene and on a selective substrate was measured by replica plating colonies obtained from NB plates onto Tol plates and onto selective plates. Replica-plated colonies on Tol plates were screened for the presence of catechol 2,3-dioxygenase (C23O) by spraying the plates with a 100 mM catechol solution. Positive colonies of *P. putida* mt-2 turn yellow due to conversion of catechol to 2-hydroxymuconic semialdehyde (HMS) (36). Mutants unable to grow on any of the selective substrates were further analyzed for the ability to grow on other substrates in batch cultures containing 1 or 5 mM substrate in MMV.

Plasmid extractions were done by using a modified method of Kado and Liu (18), as described by Duetz et al. (7). Southern hybridizations and chemiluminescent detection of plasmid DNA digested with *Eco*RI, *Sal*I, or *Xho*I were performed with a digoxigenin-labeled probe containing the promoter region of the *meta* operon (Pm) and *xylXYZ'* or containing *xylTE'* (GenBank accession number M64747) as described by the manufacturer (Boehringer, Mannheim, Germany). The probes were obtained from the plasmids pGSH3537 (2.8-kb *Sac*I-*Kpn*I fragment) and pAW31 (1.2-kb *Sal*I fragment) (5a).

The decay rate of the strains in the fed-batch culture exposed to TCE was calculated by using the formula $x_t/x_0 = e^{-a_d t}$, in which x_t is the viability on a selective plate at time t and x_0 is the viability on a selective plate at time zero, when TCE addition to the fed-batch culture started. a_d is the specific decay rate (2).

Protein profiles of the strains were made by centrifuging 60 μl of an overnight NB culture, resuspending the pellet in 10 μl of 1× loading buffer (31), and applying the mixture after boiling (5 min) on a 12.5% polyacrylamide gel containing sodium dodecyl sulfate. The gels were stained with Coomassie brilliant blue.

Enzyme assays. Mutants of *P. putida* mt-2 were analyzed for the presence of enzymes of the *meta*- and *ortho*-cleavage pathways. For this, cells were grown overnight on 1 mmol of toluene h^{-1} in a 2.5-liter fed-batch culture. Cells were harvested by centrifugation and washed with either ice-cold mineral medium (MM) or ice-cold 0.1 M Tris-HCl (pH 7.5) containing 0.1 mM 1,4-dithiothreitol (TD). After resuspension of the cells in a small volume of MM or TD, they were used for the enzyme assays, which were performed at 30°C.

The cells which were washed and resuspended in MM were used immediately for oxygen uptake experiments. For this, cells were added to a small, magnetically stirred incubation vessel to which an oxygen electrode (O$_2$ sensor type 12/220; Ingold, Urdorf, Switzerland) was connected. After determination of the endogenous oxygen consumption rate of the resting cell suspension, a concentrated solution of benzoate, *m*-toluate, or *p*-toluate was added to give a final concentration of 5 mM. The difference between the oxygen consumption rates before and after addition of the substrate was used to calculate the specific rate of oxidation of the substrate in micromoles gram of cells (dry weight)$^{-1}$ minute^{-1}.

The cells which were washed and resuspended in TD were disrupted by sonification and centrifuged for 30 min in an Eppendorf centrifuge (10,000 × g, 4°C). The clear supernatant solution was used as a source of crude cell extract. The protein content of the extract was determined with Coomassie brilliant blue, with bovine serum albumin as a standard.

Catechol 1,2-dioxygenase (C12O), C23O, 2-hydroxymuconic semialdehyde hydrolase (HMSH), and 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) activities in the crude cell extract were measured as described previously (23). The conversion of catechol, 3MC, and 4-methylcatechol (4MC) to their corresponding *meta*-cleavage products was monitored at 375, 388, and 382 nm respectively. The extinction coefficients of HMS, 2-hydroxy-6-oxohepta-2,4-dienoic acid (HODA), and 2-hydroxy-5-methylmuconic semialdehyde (HMMS) are 36,000, 16,800, and 31,500 liters mol^{-1} cm^{-1}, respectively (30). HMS, HODA, and HMMS were prepared in situ by incubation of solutions containing catechol, 3MC, or 4MC, respectively, in 45 mM phosphate buffer (pH 7.4) with purified C23O of *P. putida* mt-2 and were used to determine HMSH and HMSD activities. C23O was purified from a crude extract of *P. putida* mt-2 by using an acetone precipitation step followed by ion-exchange and hydrophobic interaction chromatographies.

Analytical methods. TCE and toluene were measured in the gas phase on a CP 9001 gas chromatograph equipped with a CP Sil 5 CB column (length, 25 m; diameter, 0.53 mm) (Chrompack, Middelburg, The Netherlands) and a flame ionization detector. The carrier gas (helium) pressure was 150 kPa, and the oven temperature was 100°C.
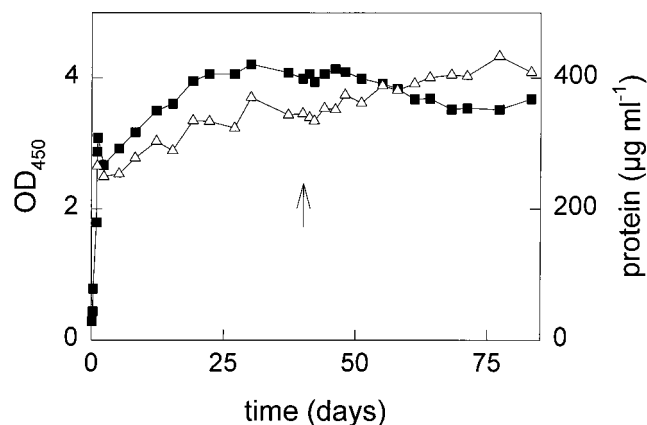
Chloride was measured with a colorimetric assay (3).

FIG. 1. Culture density of the fed-batch culture cultivated on toluene in the absence and presence of TCE. The OD and protein concentration were determined as a measure of the culture density. Symbols: ■, OD$_{450}$; △, protein concentration; ↑, time at which TCE addition was started.

Estimation of kinetic parameters. The kinetic parameters (K_s and μ_{max}) of the strains used in the mixed fed-batch culture were estimated from substrate depletion curves obtained with growing batch cultures (29). For this, the strains were grown on toluene in a 3-liter bioreactor on 0.75 liter of mineral medium at 30°C at a stirring speed of 1,050 rpm. The depletion of toluene was measured by on-line analysis of the toluene concentration in the headspace of the batch culture by gas chromatography. The headspace was continuously circulated at a rate of 100 to 120 ml min^{-1} with a micromembrane pump (NMP 02LU; KNF Neuberger GmbH, Freiburg-Munzingen, Germany). After passage through a Valco six-port sampling injector (Vici AG, Schenkon, Switzerland) to which a 35-μl sample loop was connected, the gas was injected back into the culture. Samples of 35 μl were automatically injected into the gas chromatograph every 5 min.

The obtained substrate depletion curves were described with a model in which the Monod equation and gas-liquid mass transfer of the substrate are incorporated, with biomass (X) and gas and liquid phase concentrations (C_g and C_l) as variables. The volumes of the gas and liquid phases (V_g and V_l) were 0.75 and 2.25 liters, respectively. Values between 0.048 and 0.057 mg of cells (dry weight) μmol^{-1} were taken for the yield (Y) of the different strains and were determined from batch cultures of each strain growing on 1 mM toluene. The dimensionless Henry's coefficient (H) was determined as described by Diks (6) ($H_{\text{toluene, 30°C}}$ = 0.27; $H_{\text{TCE, 30°C}}$ = 0.5). The mass transfer coefficient (k_La) for toluene was determined to be 0.14 min^{-1} by a procedure described by van Hylckama Vlieg et al. (33). The model consists of three equations: $X = X_0 + [(C_{g,0} - C_g)V_g + (C_{l,0} - C_l)V_l]Y/V_l$, $dC_g/dt = -k_La(C_g/H - C_l)V_l/V_g$, and $dC_l/dt = k_La(C_g/H - C_l) - \mu_{max}C_l/(C_l + K_s)X/Y$.

The parameters K_s, μ_{max}, and the initial concentrations of toluene in the gas and liquid phases ($C_{g,0}$ and $C_{l,0}$) were fitted to the numerically integrated equations by using the episode routine in Scientist for Windows 2.0 (Micromath Scientific Software, Salt Lake City, Utah). The square of the difference between the measured and fitted values was multiplied by $1/(C_g + 0.1)$ at each time point. The sum of these relative squares was minimized. This way, the data points at lower toluene concentrations have the same weight as the data points at higher toluene concentrations, while the more inaccurate values close to the detection limit (35 nM) are less important. The data points from the first hour were usually omitted to ensure that the system was in equilibrium.

The TCE-degrading capacities of the strains were tested with toluene-grown cells obtained from a 2-liter overnight fed-batch culture grown at a toluene addition rate of about 500 μmol h^{-1} to a density of 0.15 to 0.4 mg (dry weight) ml^{-1}. Cells were harvested by centrifugation and resuspended in MM to a final volume of 25 ml. The depletion of TCE was measured by on-line analysis of the TCE concentration in the headspace of the resting cell-suspension, which was placed in a 120-ml stainless steel incubation vessel at 30°C. TCE concentrations were determined every minute in the same way as for the toluene depletion experiments described above. The culture was stirred at 1,000 rpm. The first-order rate constant of TCE degradation, which is equal to the maximum rate of substrate conversion (r_{max}) divided by the Michaelis-Menten constant (K_m), was determined from the first-order region of the TCE depletion curve. This was done by plotting the natural logarithm of the TCE concentration in the gas phase against time and multiplying the slope of this line by $-(V_gH + V_l)/XV_l$.

RESULTS

Competition for toluene. The competitive capacities of the toluene-degrading strains *P. putida* GJ31, *P. putida* mt-2, *P. putida* F1, and *B. cepacia* G4 were studied with a mixed fed-batch culture at a very low toluene concentration. For this, cells from batch cultures of each strain were added to the fed-batch bioreactor, and the mixed culture was grown at a toluene addition rate of about 600 μmol h^{-1} until the culture reached an OD$_{450}$ of 3. The toluene load was then reduced to 44 μmol h^{-1}. Previous experiments with *B. cepacia* G4 had shown that at this combination of toluene load and culture density, the specific growth rate on toluene is very low (22).

During 2 weeks, the culture density of the mixed fed-batch culture slowly increased to an OD$_{450}$ of 4, after which it became constant (Fig. 1), meaning that hardly any net growth occurred and that all toluene added was used for maintenance of the culture. After approximately 1 week of slow growth, no toluene could be detected in the outgoing gas stream (detection limit, 35 nM), which means that more than 99.7% of the added toluene was converted (Fig. 2A) and that growth was not limited due to exhaustion of other nutrients. The dry weight of the culture was determined after 40 days, and the toluene conversion of the hardly growing, mixed fed-batch culture was calculated to be 31 nmol mg of cells (dry weight)$^{-1}$ h^{-1}.

The population composition of the culture was determined by counting the colonies on selective plates (Fig. 3A). This corresponded to the viability of each strain on NB plates, which could be determined based on colony morphology. Just after the reduction of the toluene load from about 600 to 44
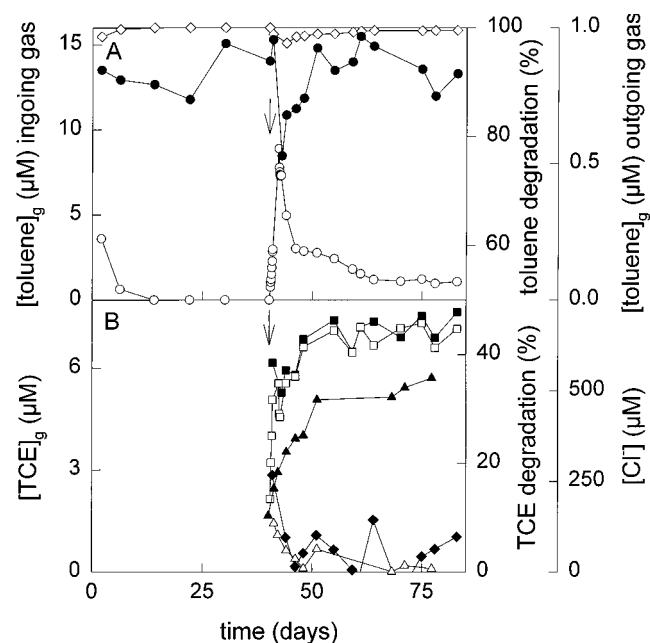

FIG. 2. Conversion of toluene (A) and TCE (B) by the fed-batch culture. The concentrations of toluene and TCE in the ingoing and outgoing gas streams were determined by gas chromatography. The chloride concentration in the culture medium was determined with a colorimetric assay. Symbols: ●, toluene in the ingoing gas stream; ○, toluene in the outgoing gas stream; ◇, percentage of toluene degraded by the culture; ■, TCE in the ingoing gas stream; □, TCE in the outgoing gas stream; ◆, percentage of TCE degraded by the culture (calculated from the concentrations of TCE in the in- and outgoing gas streams); ▲, chloride concentration; △, percentage of TCE degraded by the fed-batch culture (calculated from chloride measurements); ↓, time at which TCE addition was started.
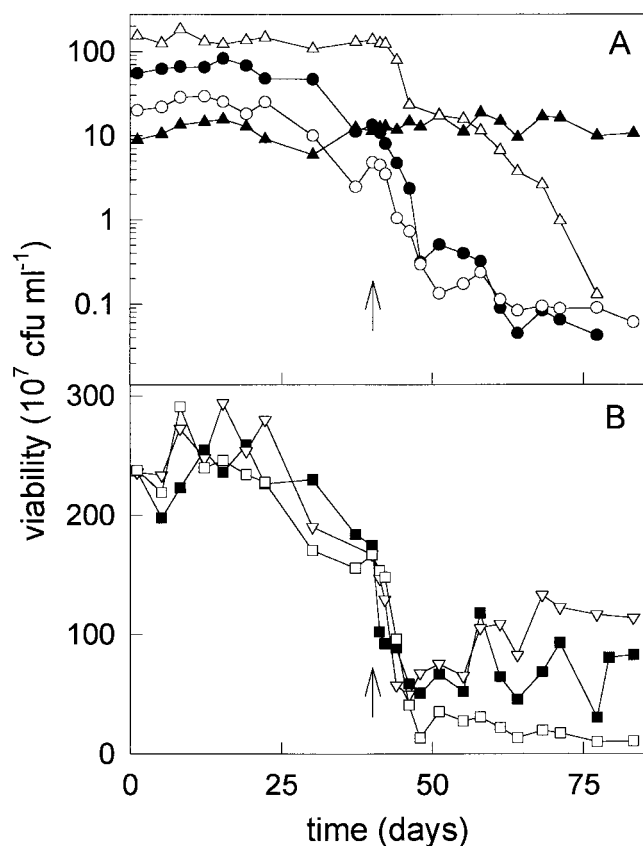
FIG. 3. Population composition (A) and viability (B) of the fed-batch culture over time, determined as CFU on different agar plates. Symbols: ●, *P. putida* GJ31 on chlorobenzene plates; ▲, *P. putida* mt-2 on *p*-xylene plates; ○, *B. cepacia* G4 on *o*-cresol plates; △, *P. putida* F1, determined as the difference between the viability on *m*-cresol and *o*-cresol plates; ■, viability on NB plates; ▽, viability on Tol plates; □, sum of viabilities of the four strains on selective plates; ↑, time at which TCE addition was started.

μmol h^{-1}, the contribution of each strain to the total viability of the mixed culture varied between 4% (for *P. putida* mt-2) and 64% (for *P. putida* F1). During the cultivation at a very low toluene concentration, the population composition hardly changed, and all four strains were maintained for at least 40 days in the culture. The sum of the viabilities of the strains on selective plates was similar to the total viability of the mixed culture, as measured on NB and Tol plates (Fig. 3B). This means that all four strains were able to grow on their selective substrate. This was confirmed by replica plating colonies derived from NB plates to selective plates.

Competition for toluene in the presence of TCE. To study the influence of TCE on the competitive behavior of the four strains at very low toluene concentrations, TCE was added to the culture at a rate of 24 μmol h^{-1}, starting after 40 days of fed-batch cultivation on toluene. During TCE addition, the culture density slowly decreased to an OD$_{450}$ of 3.6 at 83 days. Immediately after the start of TCE addition, the toluene concentration in the outgoing gas stream increased to a maximum of around 500 nM after 2 days of TCE addition. It then slowly decreased to approximately twice the detection limit (Fig. 2A). Approximately 17% of the incoming TCE was degraded after 1 day, but this became less than 3% after 4 days (Fig. 2B). The chloride concentration slowly increased in the fed-batch culture (Fig. 2B).

Within a few days after the start of the addition of TCE, the viability of *B. cepacia* G4, measured as CFU on *o*-cresol plates, decreased with a specific decay rate of 0.0037 h^{-1} ($r^2 = 0.70$). *P. putida* GJ31 and *P. putida* F1 showed similar behavior. The viability on chlorobenzene plates and the difference in viability on *m*-cresol plates and *o*-cresol plates decreased with specific decay rates of 0.0064 ($r^2 = 0.84$) and 0.0068 ($r^2 = 0.94$) h^{-1}, respectively. The viability of *P. putida* mt-2 on *p*-xylene plates remained at a constant level (Fig. 3A). These results show that only a small amount of TCE could be converted by the mixed culture and that the TCE degradation capacity decreased over time, since the TCE-converting organisms were outcompeted by *P. putida* mt-2.

Accumulation of mutants. After 1 week of TCE addition, a difference arose between the sum of the viabilities of the strains on selective plates and the viabilities of the mixed culture on NB plates and on Tol plates (Fig. 3B). To check if all the cells were still able to grow on one of the selective substrates, colonies derived from NB plates were replica plated on selective plates. An increasing amount of colonies was no longer able to grow on *p*-xylene, *o*-cresol, *m*-cresol, or chlorobenzene, indicating that these colonies are mutants.

The colony morphologies of these mutants were similar to that of *P. putida* mt-2. The protein profiles of the mutants were also found to be similar to the profile of the wild-type *P. putida* mt-2 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown), indicating that they were mutants of *P. putida* mt-2. The mutants were further examined by replica plating on Tol plates and on the selective plates. Cells grown on Tol plates were checked for C23O (XylE) activity by spraying with catechol. If XylE is active, the colonies rapidly become yellow due to the conversion of catechol to the yellow HMS.

Three classes of mutants were distinguished. One class of mutants was no longer able to grow on toluene or on any of the other selective substrates. The mutants possessed a plasmid that was much smaller than the wild-type TOL plasmid. It did not hybridize with the Pm-*xylXYZ'* and *xylTE'* probes, indicating that it lacks the 39-kb fragment encoding the catabolic genes for toluene and xylene growth. Loss of this fragment also occurs during growth on benzoate (see, e.g., reference 37). Immediately after the addition of TCE, this phenotype was observed for approximately 20% of the *P. putida* mt-2-like colonies. However, this class of mutants disappeared after 1 week (Fig. 4).

A second class of mutants was XylE$^+$ and could still grow on toluene, *m*-xylene, and *m*-toluate but not on *p*-xylene or *p*-toluate. Approximately one-third of the mutants which could still grow on toluene but not on the selective plates belonged to this class (Tol$^+$ *p*-Xyl$^-$ XylE$^+$) (Fig. 4). The specific rate at which these mutants appeared was estimated from the increase in their number over time by multiplying the number of *P. putida* mt-2-like colonies on NB plates by the percentage of Tol$^+$ *p*-Xyl$^-$ XylE$^+$ mutants, which was determined by replica plating at each time point, and was found to be 0.032 h^{-1} ($r^2 = 0.65$). No differences in plasmid size could be seen on agarose gels, and the plasmid still hybridized with the *xylTE'* and Pm-*xylXYZ'* probes. Southern hybridizations of *Eco*RI-, *Xho*I-, or *Sal*I-digested plasmid DNA with the Pm-*xylXYZ'* and *xylTE'* probes also revealed no differences. However, in a crude extract of toluene-grown cells of one of these mutants (*P. putida* mt-2M11), the activities of three enzymes of the *meta*-cleavage pathway with different substrates were approximately one-fifth of the activities found with wild-type cells (Table 2). Also, cells of *P. putida* mt-2M11 which were grown on toluene no longer oxidized *p*-toluate (Table 3). In *P. putida* mt-2, *p*-toluate is oxidized by a toluate dioxygenase (XylXYZ) which can also
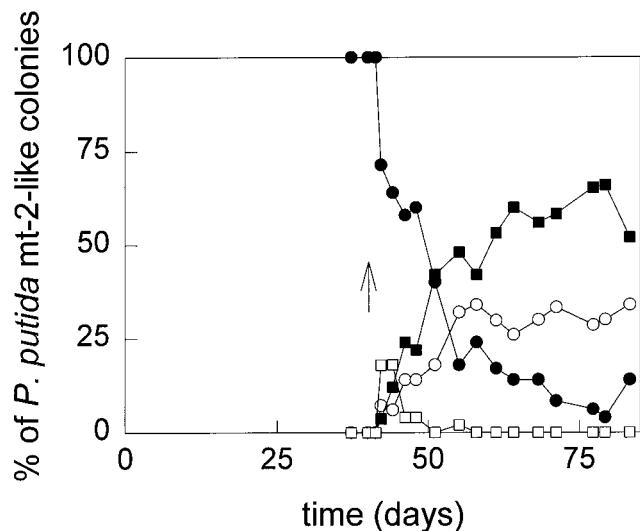
FIG. 4. Appearance of mutants of *P. putida* mt-2 during fed-batch cultivation. Colonies with a morphology similar to that of *P. putida* mt-2 on NB plates were replica plated on Tol plates and selective plates to determine their phenotype. Activity of XylE was determined by spraying Tol plates with replica-plated colonies with a catechol solution and checking for the formation of the yellow product HMS. Symbols: ●, colonies able to grow on *p*-xylene and toluene and forming HMS after being sprayed with catechol (Tol⁺ *p*-Xyl⁺ XylE⁺ [wild-type]); □, colonies unable to grow on *p*-xylene and toluene (Tol⁻ *p*-Xyl⁻); ■, colonies able to grow on toluene, unable to grow on *p*-xylene, and not forming HMS when sprayed with catechol (Tol⁺ *p*-Xyl⁻ XylE⁻); ○, colonies able to grow on toluene, unable to grow on *p*-xylene, and forming of HMS after being sprayed with catechol (Tol⁺ *p*-Xyl⁻ XylE⁺); ↑, time at which TCE addition was started.

convert benzoate and *m*-toluate. The *xylXYZ* genes are located in the *meta* operon of the TOL plasmid of *P. putida* mt-2, directly behind the promoter of the operon (1). The chromosomally encoded equivalent of XylXYZ is a benzoate dioxygenase, which can convert only benzoate and *m*-toluate (15, 28). The data suggest that the mutants of class 2 have a small mutation in the promoter region of the *meta* operon, which could not be detected by Southern analysis but which influences the expression of the *meta* pathway and leads to the absence of activity of XylXYZ.

The third class of mutants was still able to grow on toluene

TABLE 2. Specific activities of enzymes of the *ortho* and *meta* pathways in crude extracts of *P. putida* mt-2 and mutants that originated from this strain

Enzyme	Substrate	Sp act (U g of protein^{-1}) of enzyme in crude extracts of *P. putida*:			
		mt-2	mt-2M11	mt-2M10	mt-2M4
C12O	Catechol	22	29	77	55
C23O	Catechol	4,466	878	46	0
	3-MC	1,316	299	20	0
	4-MC	2,403	421	30	ND^a
HMSH	HMS	184	26.6	2.5	0
	HODA	529	85.6	4.3	ND
	HMMS	165	24.4	1.4	ND
HMSD	HMS	110	33.2	0.4	0
	HODA	46.5	4.2	0.4	ND
	HMMS	150	35.0	1.1	ND

^{a} ND, not determined.

TABLE 3. Oxygen uptake by washed resting cell suspensions of *P. putida* mt-2 and of mutants which originated from this strain

Substrate	Oxygen consumption (μmol g of cells [dry weight]$^{-1}$ min^{-1}) by cells of *P. putida*:			
	mt-2	mt-2M11	mt-2M10	mt-2M4
Benzoate	197	84	269	100
m-Toluate	112	37	22	5.7
p-Toluate	101	—^{a}	—	—

^{a} —, no activity detectable.

but not on *p*-xylene or *p*-toluate. When colonies of this class were sprayed with catechol, little or no yellow coloration occurred. The specific rate at which these Tol⁺ *p*-Xyl⁻ XylE⁻ mutants appeared was 0.064 h⁻¹ ($r^2 = 0.90$). Most of these mutants grew very poorly on *m*-xylene and *m*-toluate, while the medium became brown, which indicates the accumulation of a (substituted) catechol. One of the mutants (*P. putida* mt-2M4) was unable to grow on *m*-xylene at all. It had a plasmid which was slightly smaller than the TOL plasmid of *P. putida* mt-2 and did not hybridize with a Pm-*xylXYZ'* or a *xylTE'* probe. No activity of enzymes of the *meta* pathway could be detected (Table 2). This suggested that *P. putida* mt-2M4 lacks all or a large part of the genes of the *meta*-cleavage pathway. A similar mutation was found by Brinkmann et al. (4) during unlimited growth of *P. putida* on toluene.

The other mutants of the third phenotypic class contained a plasmid which still hybridized with both probes and seemed to have the same size as the wild-type plasmid. However, Southern analysis showed that both the *Sal*I and *Eco*RI fragments of the plasmid encoding part of *xylX* and the promoter region were about 0.4 kb smaller than the corresponding wild-type fragments. The activities of enzymes of the *meta* pathway were largely reduced in a crude extract of a toluene-grown mutant of this class (*P. putida* mt-2M10) (Table 2), indicating that the deletion caused a large reduction of the expression of the *meta* pathway. None of the mutants of class 3 could oxidize *p*-toluate (Table 3), indicating that XylXYZ is not active in these cells.

Kinetic analysis of toluene-degrading strains. To determine the kinetic basis for the observed population changes and appearance of mutants, the kinetic parameters (μ_{max} and K_s) of each strain were determined by on-line gas chromatographic analysis of toluene depletion from the headspaces of batch cultures. An example of a depletion curve is presented in Fig. 5A. The data show that *B. cepacia* G4 has a much lower affinity (μ_{max}/K_s) for toluene than the other three strains, while *P. putida* GJ31 has the best kinetics for growth on toluene (Table 4).

The TCE-degrading capacities of the strains were determined from TCE depletion curves measured with toluene-grown cells (Fig. 6). The first-order rate constants of TCE degradation were 52 and 0.6 ml mg of cells (dry weight)⁻¹ h⁻¹ for *B. cepacia* G4 and *P. putida* GJ31, respectively. For *P. putida* F1 the rate constant of TCE degradation decreased rapidly from 14 to 1.7 ml mg of cells (dry weight)⁻¹ h⁻¹. Wackett and coworkers also observed a rapid decrease of the rate over time, which was probably caused by the formation of toxic intermediates (34, 35). *P. putida* mt-2 showed no detectable TCE degradation (Fig. 6). The data show that *P. putida* mt-2 is not able to degrade TCE, while *P. putida* GJ31 degrades TCE much slower than *P. putida* F1 and *B. cepacia* G4, which are well known for their TCE-degrading capacities.

To study the effect of TCE on the kinetics of toluene utilization, toluene depletion curves were made in the presence of
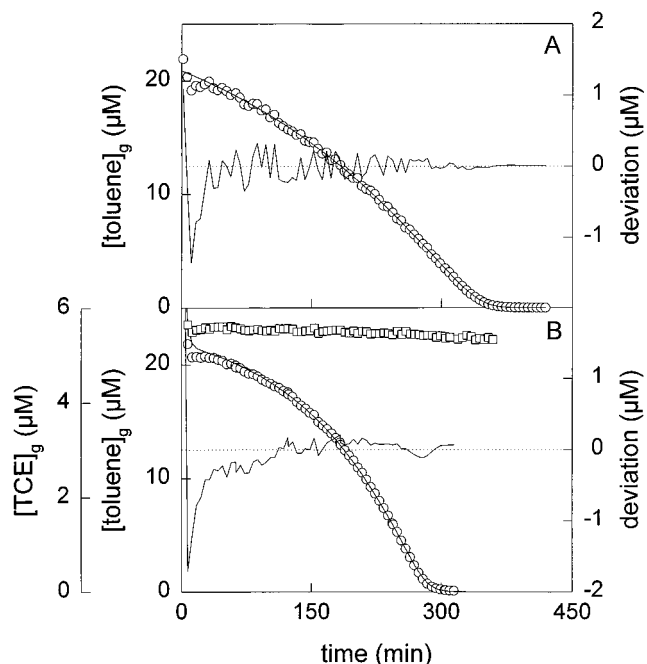
FIG. 5. Toluene depletion curves obtained with batch cultures of *P. putida* mt-2 growing on toluene in the absence of TCE (A) and *P. putida* GJ31 growing on toluene in the presence of TCE (B). The concentrations of toluene (○) and TCE (□) in the headspace of the culture were determined. The lines through the open circles are the fitted toluene depletion curves. The other lines show the deviation of the data points from the fitted curve.
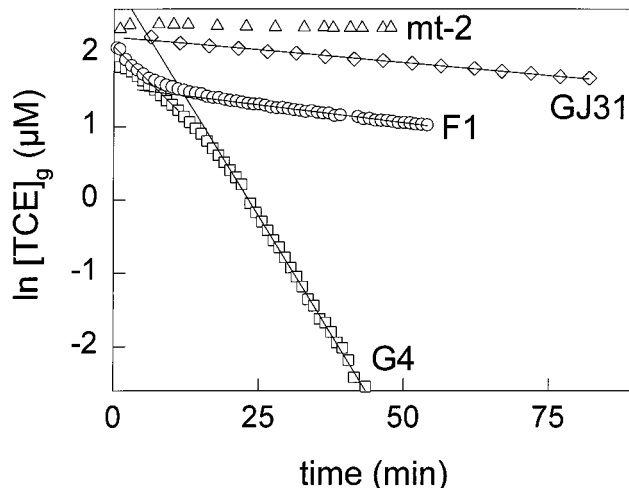

FIG. 6. Logarithmic plots of TCE depletion by resting cell suspensions of *B. cepacia* G4 and *P. putida* F1, GJ31, and mt-2. The cells were grown on toluene. The concentrations of TCE in the headspace of the cultures were measured. TCE depletion by 0.43 mg (dry weight) of *B. cepacia* G4 ml^{-1} (□), 1.0 mg (dry weight) of *P. putida* F1 ml^{-1} (○), 1.9 mg (dry weight) of *P. putida* GJ31 ml^{-1} (◇), and 3.9 mg (dry weight) of *P. putida* mt-2 ml^{-1} (△) is shown. The lines show the TCE depletion curves that were fitted through the data located in linear regions of the curves for *B. cepacia* G4, *P. putida* F1, and *P. putida* GJ31.

TCE, which was added to similar concentrations as with the fed-batch culture. *B. cepacia* G4 significantly converted TCE during growth on toluene, whereas the other three strains hardly converted TCE (Fig. 5B). The apparent affinities for toluene decreased in the presence of TCE (Table 4). For *P. putida* mt-2, this decrease was twofold. However, the affinities for toluene of *P. putida* F1 and GJ31 became almost 1 order of magnitude lower in the presence of TCE. Together with *B. cepacia* G4, these strains disappeared from the fed-batch culture after the start of TCE addition and are able to (slightly) degrade TCE.

The TCE depletion curve of *P. putida* GJ31 showed first-order behavior. This means that the half-saturation constant (K_m^{TCE}) of TCE conversion is much higher than the initial TCE concentration in the experiment, which was 18 μM in the liquid phase. TCE was present at 11 μM in the liquid phase during the preparation of the toluene depletion curve for strain GJ31. With these numbers, a model for competitive inhibition {$K_s^{obs} = K_s(1 + [TCE]/K_i)$} predicts an increase in the observed Monod constants (K_s^{obs}) with a factor of less than 1.6 if K_i were identical to K_m^{TCE}. However, the increase in K_s^{obs} which was determined from toluene depletion curves in the presence of TCE was much higher (Table 4), indicating that the inhibition of toluene utilization by TCE cannot be described by a simple model for competitive inhibition. Landa et al. (19) also found that this model could not describe cometabolic TCE degradation by *B. cepacia* G4 in continuous culture. Instead, they observed that the inhibition constant of TCE for the conversion of toluene was higher than the K_m of TCE conversion.

The kinetic parameters for growth on toluene of some mutants of *P. putida* mt-2 are given in Table 5. Mutants belonging to both class 2 and class 3 were analyzed. All five mutants had a higher μ$_{max}$ and a higher affinity than wild-type *P. putida* mt-2. The presence of TCE still caused a small decrease in the affinity for toluene (data not shown).

TABLE 4. Effect of TCE on the Monod parameters for growth on toluene[a]

Strain	Substrate(s)	K_s^{obs} (μM)	μ$_{max}$ (h^{-1})	Affinity (μ$_{max}$/K_s^{obs})
B. cepacia G4	Toluene	26.4[b]	0.30[b]	0.011
	Toluene + TCE[c]	74.7	0.39	0.005
P. putida F1	Toluene	1.5[b]	0.65[b]	0.433
	Toluene + TCE	11.2	0.60	0.054
P. putida mt-2	Toluene	3.3	0.25	0.075
	Toluene + TCE	7.3	0.28	0.038
P. putida GJ31	Toluene	0.4	0.53	1.325
	Toluene + TCE	3.7	0.53	0.143

[a] The parameters were calculated from toluene depletion curves as described in Materials and Methods.
[b] Average from two curves.
[c] TCE was added at concentrations similar to that in the fed-batch culture.

TABLE 5. Monod parameters for growth on toluene of some mutants of *P. putida* mt-2[a]

Mutant phenotype (strain)	K_s (μM)	μ$_{max}$ (h^{-1})	Affinity (μ$_{max}$/K_s)
Tol$^+$ *p*-Xyl$^-$ XylE$^+$ (mt-2M11)	2.4	0.43	0.179
Tol$^+$ *p*-Xyl$^-$ XylE$^+$ (mt-2M20)	2.9	0.42	0.145
Tol$^+$ *p*-Xyl$^-$ XylE$^-$ (mt-2M10)	3.3	0.46	0.139
Tol$^+$ *p*-Xyl$^-$ XylE$^-$ (mt-2M12)	3.7	0.47	0.127
Tol$^+$ *p*-Xyl$^-$ XylE$^-$ (mt-2M4)	5.3	0.50	0.094

[a] The parameters were calculated from toluene depletion curves as described in Materials and Methods.

DISCUSSION

The four toluene-degrading strains used in this study (*B. cepacia* G4, *P. putida* mt-2, *P. putida* GJ31, and *P. putida* F1) had to compete for very low concentrations of toluene when they were cultivated together in a fed-batch culture. In situations like this, hardly any growth of the culture is allowed. However, shifts in the population composition are still possible. Zambrano et al. (38), for example, described the takeover of a stationary culture by a mutant of *Escherichia coli*. Likewise, we observed that mutants which had lost pTOM took over a culture of *B. cepacia* G4 which was exposed to TCE while being starved for carbon and energy (22). Under the conditions that were used here, all four strains were able to maintain themselves in the fed-batch culture at a rather constant viability for at least 40 days.

The strains that were present in the fed-batch culture showed large differences in the individual kinetic parameters μ_{max} and K_s. If the capacities of the strains to compete during severe toluene limitation were determined by their kinetic properties, one would expect the strain with the highest affinity for toluene to take over the culture. Since no important changes in the population composition were observed, these kinetic parameters did not predict the outcome of the competition. This means that other factors also determined the competitive capacities of the strains. Bacteria could, for example, increase their affinity for toluene by a stronger induction of the enzymes involved in toluene degradation (20) or could produce compounds which inhibit other species. Factors like the amount of energy needed for maintenance purposes and the growth yield on products of lysed cells can also influence the survival of the strains. Also in continuous culture, the outcome of competition experiments cannot always be predicted by the affinity of each strain. Despite the lower affinity of *B. cepacia* G4 for toluene, it could win over *P. putida* mt-2 (8).

After TCE addition, only *P. putida* mt-2, which does not degrade TCE, remained in the culture. The numbers of cells of *B. cepacia* G4, *P. putida* F1, and *P. putida* GJ31 decreased, and TCE degradation diminished rapidly. We previously found that a nongrowing culture of *B. cepacia* G4 could cometabolically degrade TCE in a fed-batch culture as long as toluene was added (22). However, this resulted in a large increase in the maintenance energy demand of *B. cepacia* G4, most likely due to toxic effects of TCE conversion. Cytotoxicity by TCE conversion products has also been observed for *P. putida* F1 (35). Such toxic effects could give TCE-converting organisms a selective disadvantage, especially at the low toluene concentrations used.

Compared to that of *B. cepacia* G4, the first-order rate constant for TCE degradation of *P. putida* GJ31 suggests that at least 85 times less TCE will be converted by this strain, which is likely to be even less because of its superior kinetics for toluene degradation. In spite of that, the observed decay rates for *P. putida* GJ31 and F1 after TCE addition were even higher than the decay rate for *B. cepacia* G4. Analysis of toluene depletion curves made in the presence of concentrations of TCE similar to that in the fed-batch culture showed that the affinity (μ_{max}/K_s^{obs}) of *P. putida* GJ31 for toluene strongly decreased. Such a decrease was also observed for *P. putida* F1. For *B. cepacia* G4 the affinity decreased only 2.2-fold. However, during the kinetic measurements, *B. cepacia* G4 degraded a considerable amount of TCE, which leads to a large underestimation of K_s^{obs}.

Although the affinity for toluene is not the sole parameter determining the outcome of the competition, reduction of affinity could still lower the competitiveness of an individual strain. Quantitatively, the effect of TCE on the K_s^{obs} for toluene as determined in depletion experiments could not be described by competitive inhibition. For *P. putida* GJ31 and F1, the effect of TCE was larger than expected, which may be due to enzyme inactivation or product inhibition in addition to competitive inhibition. Our results indicate that the actual amount of TCE that can be converted by the organisms is not the only factor that determines their fate in the population, because the kinetics for the degradation of TCE did not correspond to the effect of this compound on the kinetics for growth on toluene and on the survival of the different strains.

After TCE addition was started, the competitive capacity of *P. putida* mt-2 was further improved by mutations which allowed this organism to grow on toluene at a higher rate, but this also resulted in the loss of the capacity to grow on *p*-xylene. The mutants did not convert *p*-toluate, indicating that they do not have any active XylXYZ. Also, the expression levels of enzymes further down in the *meta* pathway were strongly reduced. When the mutants grow on toluene, the enzymes of the upper pathway (1) will convert toluene to benzoate, after which the absence of XylXYZ and the reduction of the expression of the *meta* pathway probably allow benzoate to be degraded mainly via the *ortho* pathway, which is known to result in a higher growth rate (1, 4). The *p*-Xyl⁻ mutants were detected after 2 days of TCE addition (Fig. 4). By that time, the toluene concentration in the outgoing gas stream had increased to ~500 nM, probably due to the decay of *B. cepacia* G4 and *P. putida* F1 and GJ31. Since the mutants of *P. putida* mt-2 have an elevated growth rate on toluene, they could probably take over the culture more rapidly than wild-type *P. putida* mt-2.

During the 40 days of toluene addition in the absence of TCE, no *p*-Xyl⁻ mutants were detected, although they could have had an improved fitness compared to *P. putida* mt-2. Since the mutants were observed soon after the start of TCE addition, the appearance of the mutants seemed to be a direct effect of this, for example, because TCE has some mutagenic effect which increases the overall rate of mutations or because TCE specifically inhibits a component of wild-type *P. putida* mt-2. XylXYZ might be such a component, since none of the mutants of the different classes had activity of this dioxygenase, while the expression levels of other enzymes of the *meta* operon differed considerably.

Although several studies describe the potential of toluene and phenol degraders for successful remediation of TCE (10, 12–14), our results indicate that the application of microorganisms that cometabolically degrade TCE carries a high risk of takeover of the desired population by organisms that are less sensitive to inhibitory effects of TCE. Fries et al. (11) showed that there is a large variety in the capacity to degrade TCE among toluene- and phenol-degrading microorganisms isolated from the Moffett field, and they also expected that organisms which do not degrade TCE will eventually take over the population. Indeed, Munakata-Marr et al. (24) recently observed a gradual decline in the breakdown of TCE in phenol-fed microcosms containing aquifer material from the Moffett field, while degradation of phenol remained complete. This was probably caused by a shift in the population towards phenol degraders that did not degrade TCE.

Stable degradation may require the stimulation of a specific group of organisms. This might be achieved by using a less common primary substrate which can be degraded only by enzymes that also convert TCE and for which no alternatives exist. *o*-Cresol might be such a primary substrate. It is degraded by the same TCE-degrading toluene monooxygenase

(TOM) as toluene in *B. cepacia* G4 (32). TOM-containing organisms were found to dominate in the TCE-contaminated Moffett field (11), which indicates that the endogenous population can degrade TCE with *o*-cresol. In case of groundwater treatment with continuously operated bioreactors, separation of degradation and growth is another alternative to overcome instability problems and is currently under study in our lab.

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