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Published in:
Applied and environmental microbiology

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1998

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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

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Received 18 July 1997/Accepted 22 October 1997

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), 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 α-cresol to 3-methylcatechol (3MC) by two subsequent monoxygenase 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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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 center of MMY plates 1 day after plating. The plates were incubated at 30°C. Colonies appeared after 3 to 4 days. Of the five strains studied, only P. putida G3131 is able to grow on chloroform-pretreated 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 viabilities 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 1% m/m 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 chromato- nometric detection of plasmid DNA digestion with EcoRI, SacI, and SalI were performed with a digoxigenin-labeled probe containing the promoter region of the meta operon (Pm) and sigT/Y (GenBank accession number M64717) as described by the manufacturer (Boehringer, Mannheim, Germany). The probes were obtained from the plasmids pGSH3537 (2.8-kb SacI-ApaI fragment) and pAW31 (1.2-kb Sall fragment) (5a).

The decay rate of the strains in the fed-batch culture exposed to TCE was calculated by using the formula $x_{t}=x_{0} e^{-kt}$, 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. $k$ 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.

### MATERIALS AND METHODS

**Nonomenclature.** The following parameters are used in this paper: $a_{r}$, specific decay rate (minute$^{-1}$); $c$, substrate concentration (micromolar); $H$, dimensionless Henry’s coefficient; $k_{s, a}$, mass transfer rate coefficient (minute$^{-1}$); $K_{i}$, inhibition constant (micromolar); $K_{m}$, Michaelis-Menten constant (micromolar); $K_{n}$, Monod constant (micromolar); $r_{max}$, maximal specific growth rate (minute$^{-1}$); $r_{max, s}$, 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 $r_{max}$).

**TABLE 1. Substrate supply parameters**

<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>Value in fed-batch culture grown on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toluenene + TCE (0-40 days)</td>
</tr>
<tr>
<td>Airflow (ml min$^{-1}$)</td>
<td>53.2</td>
</tr>
<tr>
<td>Toluene flow (ml min$^{-1}$)</td>
<td>20.0</td>
</tr>
<tr>
<td>TCE flow (ml min$^{-1}$)</td>
<td>22.5</td>
</tr>
<tr>
<td>[Toluene] in going gas stream (µM)</td>
<td>13.3</td>
</tr>
<tr>
<td>[TCE] in going gas stream (µM)</td>
<td>7.1</td>
</tr>
</tbody>
</table>

* 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 organ- isms, which caused a loss of the TCE degradation capacity.
Estimation of kinetic parameters. The kinetic parameters (\(K_{\text{m}}\) and \(\mu_{\text{max}}\)) 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 1,000 rpm. The TCE concentration 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 Valse six-port sampling injector (Viel 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 (\(C_{\text{g}}\) and \(C_{\text{l}}\)) as variables. The volumes of the gas and liquid phases (\(V_{\text{g}}\) and \(V_{\text{l}}\)) were 0.75 and 2.25 liters, respectively, Values between 0.048 and 0.057 mg of cells (dry weight) \(\mu\)mol\(^{-1}\) 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 (\(k_{\text{La}}\)) 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: \(X = X_{\text{c}} + \left[C_{\text{g}} - C_{\text{c}}\right]V_{\text{g}} - \left[C_{\text{c}} - C_{\text{l}}\right]V_{\text{l}}\) and \(dC_{\text{g}}/dt = -k_{\text{La}}(C_{\text{g}}H - C_{\text{c}}) - \mu_{\text{max}}C_{\text{c}}(C_{\text{c}} + H)X/Y\).

The parameters \(K_{\text{c}}, \mu_{\text{max}}\), and the initial concentrations of toluene in the gas and liquid phases (\(C_{\text{g},0}\) and \(C_{\text{l},0}\)) 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 \(1/(C_{\text{c}} + 0.1)\) 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 \(\mu\)mol h\(^{-1}\) until the culture reached an OD\(_{500}\) of 3. The toluene load was then reduced to 44 \(\mu\)mol 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\(_{500}\) 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. 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\(_{500}\); △, protein concentration; □, time at which TCE addition was started.

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.

RESULTS

Competitive 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 \(\mu\)mol h\(^{-1}\) until the culture reached an OD\(_{500}\) of 3. The toluene load was then reduced to 44 \(\mu\)mol h\(^{-1}\).
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 EcoRI-, _Xho_1-, or SalI-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. 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.](image-url)
convert benzoate and m-toluolate. 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-toluolate (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 but not on p-xylene or p-toluolate. 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 (r² = 0.90). Most of these mutants grew very poorly on m-xylene and m-toluolate, 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* and *EcoR* 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 m-toluolate. Deletion caused a large reduction of the expression of the *meta* pathway (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*) 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/Kₐ) 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. Wackert 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
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 ($K_{s,TE}$) 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 ($K_{o,obs} = K_m (1 + [TCE] K_{TE})$) predicts an increase in the observed Monod constant ($K_{o,obs}$) with a factor of less than 1.6 if $K_m$ were identical to $K_{s,TE}$. However, the increase in $K_{o,obs}$ 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**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate(s)</th>
<th>$K_{o,obs}$ (μM)</th>
<th>$μ_{max}$ (h⁻¹)</th>
<th>Affinity ($μ_{max}/K_{o,obs}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cepacia</em> G4</td>
<td>Toluene</td>
<td>26.4a</td>
<td>0.30b</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Toluene + TCE</td>
<td>74.7</td>
<td>0.39</td>
<td>0.005</td>
</tr>
<tr>
<td><em>P. putida</em> F1</td>
<td>Toluene</td>
<td>1.5b</td>
<td>0.65b</td>
<td>0.433</td>
</tr>
<tr>
<td></td>
<td>Toluene + TCE</td>
<td>11.2</td>
<td>0.60</td>
<td>0.054</td>
</tr>
<tr>
<td><em>P. putida</em> mt-2</td>
<td>Toluene</td>
<td>3.3</td>
<td>0.25</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>Toluene + TCE</td>
<td>7.3</td>
<td>0.28</td>
<td>0.038</td>
</tr>
<tr>
<td><em>P. putida</em> GJ31</td>
<td>Toluene</td>
<td>0.4</td>
<td>0.53</td>
<td>1.325</td>
</tr>
<tr>
<td></td>
<td>Toluene + TCE</td>
<td>3.7</td>
<td>0.53</td>
<td>0.143</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>Mutant phenotype (strain)</th>
<th>$K_m$ (μM)</th>
<th>$μ_{max}$ (h⁻¹)</th>
<th>Affinity ($μ_{max}/K_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tol⁺ p-Xyl⁺ XyIE⁺ (mt-2M11)</td>
<td>2.4</td>
<td>0.43</td>
<td>0.179</td>
</tr>
<tr>
<td>Tol⁺ p-Xyl⁺ XyIE⁺ (mt-2M20)</td>
<td>2.9</td>
<td>0.42</td>
<td>0.145</td>
</tr>
<tr>
<td>Tol⁺ p-Xyl⁺ XyIE⁺ (mt-2M10)</td>
<td>3.3</td>
<td>0.46</td>
<td>0.139</td>
</tr>
<tr>
<td>Tol⁺ p-Xyl⁺ XyIE⁺ (mt-2M12)</td>
<td>3.7</td>
<td>0.47</td>
<td>0.127</td>
</tr>
<tr>
<td>Tol⁺ p-Xyl⁺ XyIE⁺ (mt-2M4)</td>
<td>5.3</td>
<td>0.50</td>
<td>0.094</td>
</tr>
</tbody>
</table>

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 \( \mu_{\text{max}} \) 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 a stronger induction of the enzymes involved in toluene degradation (20) or could produce compounds which inhibit 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-toluene, indicating that they do not have any active XylXZY. 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 \( \sim 500 \text{ nM} \), 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. F1. 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 monoxygenase.

finity could still lower the competitiveness of an individual strain. Quantitatively, the effect of TCE on the \( K_s \) of 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. A linear relationship between the competitive capacities of the strains. Bacter-
(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.

**ACKNOWLEDGMENTS**

This work was financed by grants from the Dutch IOP Environmental Biotechnology program and the EC environment program.

We acknowledge Uwe Dehmel for providing the plasmids with the *xylE*′ and *pm-xyLYZ*′ genes, Wouter Duetz assisted with the *P. putida* mt-2 plasmid isolations.

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