

## Degradation of Toluene and Trichloroethylene by *Burkholderia cepacia* G4 in Growth-Limited Fed-Batch Culture

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*Burkholderia (Pseudomonas) cepacia* G4 was cultivated in a fed-batch bioreactor on either toluene or toluene plus trichloroethylene (TCE). The culture was allowed to reach a constant cell density under conditions in which the amount of toluene supplied equals the maintenance energy demand of the culture. Compared with toluene only, the presence of TCE at a toluene/TCE ratio of 2.3 caused a fourfold increase in the specific maintenance requirement for toluene from 22 to 94 nmol mg of cells (dry weight)<sup>-1</sup> h<sup>-1</sup>. During a period of 3 weeks, approximately 65% of the incoming TCE was stably converted to unidentified products from which all three chlorine atoms were liberated. When toluene was subsequently omitted from the culture feed while TCE addition continued, mutants which were no longer able to grow on toluene or to degrade TCE appeared. These mutants were also unable to grow on phenol or *m*- or *o*-cresol but were still able to grow on catechol and benzoate. Plasmid analysis showed that the mutants had lost the plasmid involved in toluene monooxygenase formation (pTOM). Thus, although strain G4 is much less sensitive to TCE toxicity than methanotrophs, deleterious effects may still occur, namely, an increased maintenance energy demand in the presence of toluene and plasmid loss when no toluene is added.

Trichloroethylene (TCE) is widely used as a solvent and a degreasing agent. It has been spilled in the environment and causes concern because of its toxicity and possible carcinogenic properties (10, 11). No microorganisms which can grow on this compound are known, but cometabolic conversion of TCE by oxygenases has been described for several types of microorganisms (6). *Burkholderia (Pseudomonas) cepacia* G4 can aerobically degrade TCE when grown on toluene or other aromatic compounds as carbon and energy source (17, 18). Toluene is oxidized to *o*-cresol and subsequently to 3-methylcatechol by a toluene monooxygenase (TomA). This enzyme is also responsible for the oxidation of TCE (24, 25). Similarly, methanotrophic bacteria can convert TCE cometabolically by a soluble methane monooxygenase (9, 21, 29).

Compared with methanotrophic TCE degraders, *B. cepacia* G4 is more resistant to high TCE concentrations and shock loadings (14). The organism has a first-order rate constant of TCE degradation similar to that of methanotrophic TCE degraders (8, 14, 20) and seems a suitable candidate for application in bioreactors for removing TCE, since higher specific degradation rates can be obtained in a continuous reactor (14).

When organisms mediating cometabolic conversions are applied for contaminant removal in bioreactors with immobilized organisms, such as trickling filters, excess growth substrate might lead to large quantities of biomass which can clog the system (5). It has also been shown that an aromatic growth substrate can be a competitive inhibitor of TCE conversion by *B. cepacia* G4 (8, 14). Therefore, TCE conversion under non-growth conditions in which the concentration of the aromatic growth substrate is minimal would be most desirable, i.e., conditions in which the substrate supply meets the minimal need of the culture for maintaining its activity and cell density. The behavior of *B. cepacia* G4 under such conditions has not yet been investigated, however.

Bacteria can be cultivated without net growth of cells in a fed-batch reactor in which the growth-limiting substrate is added at a constant rate. The cells will multiply until the culture density becomes so high that the culture is no longer able to grow with the amount of substrate supplied, because all substrate is used for maintenance (7). In this paper, the TCE degradation capacity of *B. cepacia* G4 in such a growth-limited fed-batch culture is described. Toluene was used as the primary substrate and was, like TCE, added via the gas phase. The results show that *B. cepacia* G4 could stably degrade TCE under nongrowth conditions. However, the presence of TCE led to a large increase in the toluene demand per amount (dry mass) of cells of *B. cepacia* G4 maintained in the culture. When toluene addition was stopped, TCE degradation dropped, and the culture lost monooxygenase activity because of plasmid loss.

### MATERIALS AND METHODS

**Bacterial strain and culture conditions.** *B. cepacia* G4 (18) was a gift from M. S. Shields, U.S. Environmental Protection Agency, Gulf Breeze, Fla. The organism was 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<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O, 1.4 g of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.0 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 ml of a trace element solution, and 1 ml of a vitamin solution (12). 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 regulated at 7.2 with autoclaved 0.5 M NaOH and 0.25 M H<sub>2</sub>SO<sub>4</sub>. The temperature was set at 28°C, and the impeller speed was set at 1,500 rpm. To prevent formation of foam, the impeller speed was reduced to 1,000 rpm during the period in which the fed-batch culture was depleted of toluene.

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<sup>-1</sup>, 0 to 20 ml min<sup>-1</sup>, and 0 to 500 ml min<sup>-1</sup>; Bronkhorst High-Tec B.V., Veenendaal, The Netherlands). The outgoing gas stream was led through a water column of 0.5 m to set the fermentor under slight overpressure, which facilitated the detection of possible leakages.

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TABLE 1. Substrate supply parameters

Parameter	Fed-batch culture grown on:	
	Toluene	Toluene + TCE
Airflow (ml min <sup>-1</sup> )	53.5	46.2
Toluene flow (ml min <sup>-1</sup> )	2.0	2.1
TCE flow (ml min <sup>-1</sup> )		22.4
[Toluene], ingoing gas stream (μM) <sup>a</sup>	13.3	13.4
[TCE], ingoing gas stream (μM) <sup>a</sup>		6.0

<sup>a</sup> Average taken from gas chromatography measurements.

**Analysis of the culture.** 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 5 to 10 μl of pure toluene (Tol plates) was added on a paper filter disk in the cover of the petri dish. The plates were incubated at 30°C. Colonies on rich plates appeared after 2 days, and those on Tol plates were seen after 4 to 5 days.

The percentage of cells that formed colonies on NB plates and were also able to grow on toluene was measured by replica plating colonies obtained from NB plates onto Tol plates. Mutants unable to grow on toluene were further analyzed for the ability to grow on other substrates in batch cultures containing 1 mM substrate in MMV.

Cometabolic TCE conversion was tested by adding a mixture of 0.25 mM TCE and 1 mM filter-sterilized phenol solution to a culture pregrown on 20 mM citrate–1 mM phenol. The chloride concentration was measured (see below) as an indication of TCE conversion. Wild-type cells of *B. cepacia* G4 were used as a control.

Plasmid extractions were done according to a modified method of Kado and Liu (13), as described by Rohde (23).

The culture density was estimated by measuring the optical density at 450 nm (OD<sub>450</sub>) on a Hitachi 100-60 spectrophotometer, by determining the protein concentration according to Lowry et al. (16) (with bovine serum albumin as a standard), and by determining the dry weight of the culture. The last procedure was done by centrifuging 200 ml of culture (15 min, 6,000 × g, 4°C), washing the pellet with the same volume of cold demineralized water, and drying the washed pellet to a constant weight in a preweighed aluminum cup for 2 to 3 days at 80°C.

The decay rate of cultures starved for toluene was calculated by using the formula  $x_t/x_0 = e^{-a_d t}$ , in which  $x_t$  is the amount of cells at time  $t$  and  $x_0$  is the amount of cells at time zero, when the culture was set to complete starvation for toluene.  $a_d$  is the specific decay coefficient (hour<sup>-1</sup>) (1).

**Analytical methods.** TCE and toluene were measured in the gas phase on a Chrompack 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 pressure (N<sub>2</sub>) was 150 kPa, and the oven temperature was 100°C.

Chloride was measured by a colorimetric assay (2).

## RESULTS

**Fed-batch culture of *B. cepacia* G4 with toluene.** To determine the toluene demand of a nongrowing culture of *B. cepacia* G4, the organism was cultivated in a 2.5-liter fed-batch reactor with toluene as the growth-limiting substrate. Toluene was supplied at a rate of approximately 300 μmol h<sup>-1</sup> until, after 1 week, the culture reached an OD of 3.0, after which the toluene load was reduced to 45 μmol h<sup>-1</sup> (for substrate supply parameters, see Table 1). Previous experiments had shown that at this toluene load about a 50% further increase of the cell density could be expected. The OD of the culture became constant after about 4 weeks (Fig. 1A). The dry weight of the culture was determined, and the toluene conversion of the culture under nongrowing conditions was calculated to be 22 nmol mg of cells (dry weight)<sup>-1</sup> h<sup>-1</sup>. Hardly any toluene could be detected in the outgoing gas stream (detection limit, ±100 nM).

The viability of the culture was determined by counting the amount of colonies on rich plates (NB plates) and on mineral plates to which toluene was added (Tol plates). The viability reached a constant level after approximately 2 weeks (Fig. 1B). After about 4 weeks, the viability of the culture on Tol plates

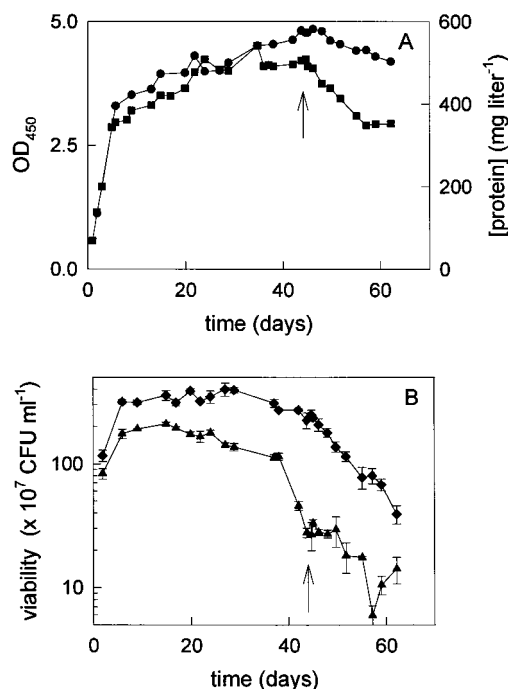


FIG. 1. Culture density of fed-batch culture of *B. cepacia* G4 with toluene in time, measured as OD<sub>450</sub> (■) and protein concentration (●) (A), and viability on NB (◆) and Tol (▲) agar plates (B). ↑, time at which the toluene feed was stopped.

became two- to fivefold lower than that on NB plates. Colonies from NB plates were replica plated to see whether they were still able to grow on toluene. No colonies which were unable to grow on toluene were found. Since all tested colonies were able to grow on toluene, the observed difference in viability between NB plates and Tol plates probably results from a difference in the capacity of cells cultivated under very low substrate concentrations to form colonies on NB plates and Tol plates. Impaired viability on selective plates of cells cultivated under carbon-limited conditions has been observed before (15).

After a period of about 2 weeks in which the OD of the culture barely changed, the supply of toluene was stopped to study the effect of complete starvation on the measured parameters. The viability on NB and Tol plates decreased with specific decay rates of 0.0040 ( $r^2 = 0.98$ ) and 0.0026 ( $r^2 = 0.81$ ) h<sup>-1</sup>, respectively. All replica-plated colonies from the NB plates were able to grow on toluene. The specific rate at which the protein concentration decreased was 1 order of magnitude lower than the decay rate of viability (Table 2). The cell density decreased from 0.79 mg (dry weight) ml<sup>-1</sup> at the onset of starvation to 0.58 mg (dry weight) ml<sup>-1</sup> after 18 days of starvation.

**Fed-batch culture of *B. cepacia* G4 with toluene and with TCE as cosubstrate.** To study the possibility of continuous TCE degradation under nongrowth conditions, *B. cepacia* G4 was cultivated in a 2.5-liter fed-batch reactor with toluene as the growth-limiting substrate in the presence of TCE. The culture was grown on about 300 μmol of toluene h<sup>-1</sup> until it reached an OD of 1.0. Then, the toluene load was reduced to 57 μmol h<sup>-1</sup> and TCE addition was started at a load of 25 μmol h<sup>-1</sup> (Table 1). The culture OD became constant after about 11 days (Fig. 2A).

The removal of toluene and TCE from the supplied gas mixture was followed by gas chromatography. An increasing

TABLE 2. Influence of TCE on *B. cepacia* in fed-batch culture

Characteristic	Fed-batch culture grown on:	
	Toluene	Toluene + TCE
Biomass concn (g liter <sup>-1</sup> )	0.791	0.242
Culture vol (ml)	2,500	2,500
Specific rate of toluene degradation (nmol mg of cells <sup>-1</sup> h <sup>-1</sup> )	22	94
Volumetric activity for TCE (nmol liter <sup>-1</sup> min <sup>-1</sup> )		106
Initial decay rate (h <sup>-1</sup> )		
Viability on NB plates	0.0040 ( $r^2 = 0.98$ )	0.028 ( $r^2 = 0.97$ )
Viability on Tol plates	0.0026 ( $r^2 = 0.81$ )	0.025 ( $r^2 = 0.90$ )
Protein concn	0.00032 ( $r^2 = 0.95$ )	0.0019 ( $r^2 = 0.95$ )

amount of TCE was degraded by the culture until a maximum of approximately 65% was reached after about 1 week of TCE addition (Fig. 3A). Chloride measurements showed that all three chlorine atoms of TCE were liberated as chloride (Fig. 3B). The specific TCE degradation rate was calculated to be 26.5 nmol mg of cells (dry weight)<sup>-1</sup> h<sup>-1</sup>. Hardly any toluene could be detected in the outgoing gas stream before TCE addition started. When the TCE flow was switched on, the toluene concentration in the outgoing gas stream initially increased but then slowly decreased to values around the detection limit (Fig. 3A), resulting in a molecular TCE/toluene conversion ratio of 0.28.

The steady-state culture density obtained in the presence of

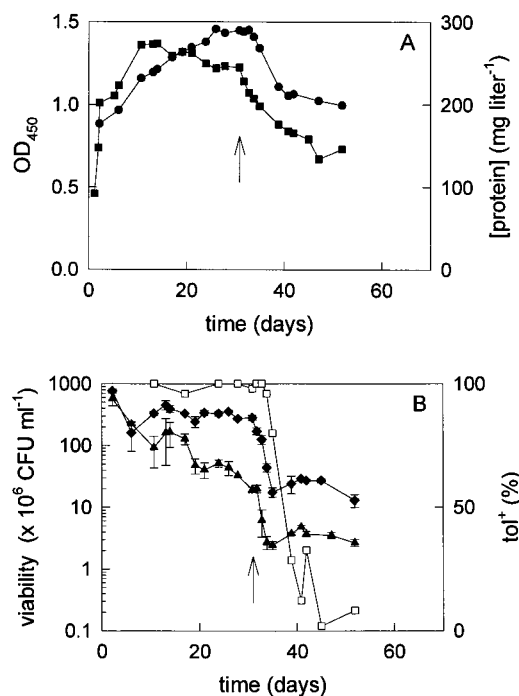


FIG. 2. Viability of fed-batch culture of *B. cepacia* G4 with toluene and TCE in time, measured as OD<sub>450</sub> (■) and protein concentration (●) (A), and viability on NB (◆) and Tol (▲) agar plates (B). □, percentage of colonies on NB plates able to grow on toluene; ↑, time at which the toluene feed was stopped.

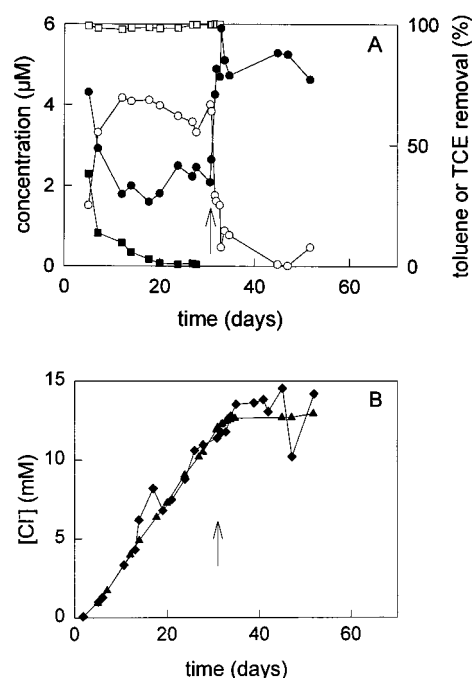


FIG. 3. Concentration of toluene (■) and TCE (●) in the outgoing gas stream and percentage of toluene (□) and TCE (○) degraded by the fed-batch culture with toluene and TCE (A), and measured (◆) and calculated (▲) chloride concentrations in the fed-batch culture (B). The calculated chloride concentration was determined from gas chromatography measurements, assuming that three chloride ions were liberated from each molecule of TCE removed. ↑, time at which the toluene feed was stopped.

TCE was about one-fourth of the final density of the culture without TCE (Fig. 2A). Thus, TCE addition caused a strong decrease in the amount of biomass that could be produced from toluene. The dry weight of the culture was determined after the culture had been exposed to toluene and TCE for about 4 weeks. From this, the maintenance energy demand, i.e., the specific rate of toluene conversion required to maintain a culture with a constant cell density, was calculated to be 94 nmol mg of cells (dry weight)<sup>-1</sup> h<sup>-1</sup>, which is fourfold higher than that in the absence of TCE (Table 2).

Viability measurements gave two- to eightfold higher counts (CFU per milliliter) on NB plates than on Tol plates (Fig. 2B). Colonies from NB plates were replica plated to see whether they were still able to grow on toluene. During the period of toluene addition, only 3 of 250 colonies tested could not grow on toluene (Fig. 2B). Thus, most cells which formed colonies on NB plates are still able to grow on toluene, although their ability to form colonies on toluene plates has decreased. This was also observed in the fed-batch culture with toluene in the absence of TCE.

In order to study the effect of TCE on the activity and stability of *B. cepacia* G4 during toluene starvation, the toluene supply was switched off after 4 weeks of simultaneous toluene and TCE degradation in the fed-batch culture. TCE degradation ceased within 3 days, and the chloride concentration in the culture no longer increased (Fig. 3).

The viability decreased, as was observed in the experiment without TCE during starvation. However, the initial decay rate was 1 order of magnitude faster than the decay rate observed in the absence of TCE (Table 2). After about 1 week, the viability no longer decreased that rapidly (Fig. 2B). This pattern of initial rapid decay which ceases after about 1 week was

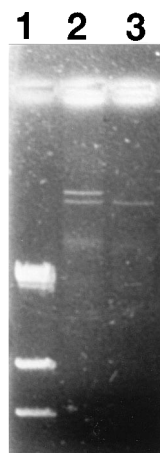


FIG. 4. Plasmid profiles of wild-type and mutant *B. cepacia* G4 cells. Lanes: 1,  $\lambda$   $\times$  HindIII; 2, wild type; 3, Tol<sup>-</sup> mutant.

also observed in the determination of culture density. The protein concentration rapidly dropped at first, with a specific decay rate of  $0.0019 \text{ h}^{-1}$  ( $r^2 = 0.95$ ), but this rate decreased to  $0.00028 \text{ h}^{-1}$  ( $r^2 = 0.99$ ) after about 8 days. The latter value is comparable to the protein decay rate determined in the culture starved for toluene in the absence of TCE (Table 2). At that time, the measured percentage of cells that did not utilize toluene (Tol<sup>-</sup>) fluctuated between 67 and 98%. The Tol<sup>-</sup> cells never completely took over the culture, because the increased decline of viability on Tol plates stopped after about 8 days. The cell density decreased from  $0.24 \text{ mg (dry weight) ml}^{-1}$  at the onset of starvation to  $0.15 \text{ mg (dry weight) ml}^{-1}$  after 21 days of starvation.

Replica plating colonies derived from NB plates showed that after 3 days of starvation in the presence of TCE, colonies which were no longer able to grow on toluene appeared on NB plates (Fig. 2B). The specific rate at which these Tol<sup>-</sup> mutants appeared in the culture was estimated to be  $0.016 \text{ h}^{-1}$  ( $r^2 = 0.97$ ) by multiplying the viability on NB plates by the percentage of Tol<sup>-</sup> colonies, determined by replica plating.

**Analysis of mutants.** Eleven Tol<sup>-</sup> mutants were streaked on NB plates and further analyzed. All mutants behaved identically in the growth tests performed. In contrast to wild-type *B. cepacia* G4 cells, the mutants were not able to grow on phenol, *m*-cresol, *o*-cresol, or toluene and did not liberate chloride when phenol and TCE were added to a culture pregrown on 20 mM citrate–1 mM phenol. When grown on benzoate and catechol, the culture of wild-type cells became yellow, in contrast to the mutant cells, which did not produce yellow compounds.

Recently, the largest of the two plasmids present in *B. cepacia* G4 has been shown to be involved in toluene degradation (26). A plasmid isolation procedure performed with *B. cepacia* G4 showed the two plasmid bands on a 0.8% agarose gel. Four mutants were tested for the presence of these plasmids. The upper band had disappeared, indicating that plasmid loss had occurred (Fig. 4).

## DISCUSSION

When *B. cepacia* G4 is grown in a fed-batch culture on toluene, it eventually reaches a constant cell density at which the amount of substrate added equals the maintenance energy demand of the culture under these nongrowth conditions (7). When microorganisms are used for biological treatment purposes, degradation under nongrowth ("maintenance") condi-

TABLE 3. Comparison of TCE transformation characteristics of *B. cepacia* G4 in continuous culture<sup>a</sup> and in fed-batch culture

Characteristic	Continuous culture	Fed-batch culture
Toluene/TCE ratio added	2.3	2.3
Toluene conversion (%)	71	>99
Specific toluene consumption (nmol mg of cells <sup>-1</sup> h <sup>-1</sup> )	2,250	94
TCE conversion (%)	7	65
Specific TCE conversion rate (nmol mg of cells <sup>-1</sup> h <sup>-1</sup> )	96.6	26.5
Conversion ratio (nmol of TCE nmol of toluene <sup>-1</sup> )	0.04	0.28

<sup>a</sup>  $D = 0.08 \text{ h}^{-1}$  (14).

tions is very suitable, because clogging of bioreactors and formation of excess biomass waste are prevented; also, the use of growth substrate is not necessary. In addition, a situation of very low substrate availability is very common in nature, and microorganisms used for in situ bioremediation will probably frequently face these circumstances. A fed-batch culture in which substrate addition occurs via the gas phase is a very elegant tool for studying the behavior of the microorganisms under maintenance conditions. Addition of substrate via the gas phase is preferable to addition of substrate in the liquid phase because it can be supplied as a continuous flow instead of a dropwise addition, which is difficult to distribute equally over the culture. With halogenated substrates, a fed-batch culture has the disadvantage of accumulating halide ions in the medium, which eventually can become inhibitory. For a long-term study, a recycle fermentor, in which the medium is refreshed while containing the cells (4), could be an alternative for studying microorganisms under maintenance conditions. This also prevents the depletion of vitamins or other nutrients.

The fed-batch culture of *B. cepacia* G4 on toluene and TCE shows that this organism is capable of stable toluene and TCE degradation under nongrowth conditions. During more than 3 weeks of simultaneous addition of toluene and TCE at a ratio of 2.3, about 65% of the ingoing TCE was converted to unidentified products and chloride in the fed-batch culture. Previously, we studied TCE degradation in a rapidly growing continuous culture of *B. cepacia* G4 (14). The percentage of TCE which was degraded in the fed-batch culture is almost 10 times higher than in continuous culture at this toluene/TCE ratio (Table 3) (14). Also, from the large difference in conversion percentages for toluene and in the TCE/toluene conversion ratios (Table 3), it can be concluded that the efficiency at which TCE was degraded is much higher under nongrowing conditions. We already showed that the TCE conversion percentage in continuous culture increased when the growth rate decreased (14). Not all of the TCE was degraded by the fed-batch culture. The TCE removal can be optimized by increasing the toluene/TCE ratio (14).

The maintenance energy demand of *B. cepacia* G4 fed with toluene in the nongrowing fed-batch culture can be determined by dividing the amount of substrate added by the amount of cells present (7). We found a value of  $22 \text{ nmol mg of cells}^{-1} \text{ h}^{-1}$ . The maintenance coefficient of *B. cepacia* G4 on phenol was determined under growing conditions by Solomon et al. (28) in continuous culture, using the model of Pirt (22) and a modified model. Their value was at least 10-fold higher than our value for toluene demand in the nongrowing fed-batch culture. The amount of energy available from the oxidation of phenol to CO<sub>2</sub> via the citric acid cycle is about 85% of the amount of energy available from toluene, which cannot

explain the observed difference between the maintenance coefficients. It has often been observed that the maintenance energy demand depends on the growth rate of the cells (3). van Verseveld et al. (31) observed a large discrepancy between the maintenance coefficients of *Paracoccus denitrificans* measured in continuous culture and that measured in a recycle fermentor, which is comparable to a fed-batch culture. Only when the specific growth rate of the strain in the recycle fermentor was higher than about  $0.01 \text{ h}^{-1}$  was the maintenance coefficient similar to the value previously determined in continuous culture (30). At lower growth rates, a linear increase in biomass was observed in the recycle fermentor, which suggests a constant growth yield and leads to a much lower maintenance coefficient. Thus, the difference in maintenance coefficients of *B. cepacia* G4 grown on toluene and phenol probably results from the differences in growth conditions.

The addition of TCE to the fed-batch culture of *B. cepacia* G4 led to a fourfold decrease in final cell dry weight. This indicates that under nongrowing conditions the conversion of TCE results in a large increase in the energy needed to maintain a certain culture density. During growth in continuous culture ( $D = 0.08 \text{ h}^{-1}$ ), a 25% decrease of the yield on toluene was observed at this toluene/TCE ratio, which probably also resulted from a large increase in maintenance energy demand of the culture (14).

The cometabolic degradation of TCE by *B. cepacia* G4 is likely to cost the organism extra energy. TomA, which oxidizes toluene to *o*-cresol and 3-methylcatechol and is also responsible for TCE conversion, needs reducing power as a cofactor (19, 25). Converting TCE to its corresponding epoxide, which is believed to be the first step in TCE degradation by monooxygenases, is likely to require reducing equivalents as well (6, 32). However, if one assumes that the conversion of one molecule of TCE requires two electrons in the form of NADH, the observed transformation of TCE can account only for 2% of the total amount of reducing equivalents obtained from toluene when toluene is fully degraded to  $\text{CO}_2$  by the citric acid cycle. Thus, the extra energy cost by TCE conversion itself cannot explain the large increase of the maintenance energy demand caused by TCE.

We suggest that toxic effects are the main cause of the increase. Cell damage by nonspecific reactions of TCE conversion products with cell components has been observed in cultures of *Methylosinus trichosporium* OB3b (20) and *Pseudomonas putida* F1 (33). The more rapid decline of the viability and protein concentration in the culture completely starved for toluene in the presence of TCE and the appearance of mutants which could no longer grow on toluene, or degrade TCE, indicate that TCE also has a negative effect on *B. cepacia* G4 cells. A toluene dioxygenase mutant of *P. putida* F1 which was unable to degrade TCE no longer showed toxic effects from the presence of TCE (33), so the formation of mutations which lead to the inability to convert TCE could be the result of cells adapting to situations in which TCE conversion causes damage.

During growth of *B. cepacia* G4 on catechol or benzoate, a yellow product accumulated in the medium, indicating a conversion via catechol-2,3-dioxygenase (*meta* route) to 2-hydroxymuconic semialdehyde (27). Recently, Shields et al. (26) demonstrated that the first two genes involved in the degradation of toluene (*tomA* and *tomB*) are localized on the largest of the two plasmids present in *B. cepacia* G4 (pTOM). *tomA* and *tomB* encode a toluene monooxygenase and a catechol-2,3-dioxygenase, respectively. The Tol<sup>−</sup> mutants which appeared in the fed-batch culture with TCE in the absence of toluene lack pTOM. They are still able to grow on catechol or benzoate

but do not accumulate a yellow product. This probably means that they degrade these compounds via an *ortho* route, which yields the colorless muconate (27) and is not encoded on pTOM. Shields et al. (25) already suggested that *B. cepacia* G4 also possesses an *ortho* route for the degradation of aromatic compounds.

Whereas the culture stably degraded TCE for 3 weeks when toluene was added, a rapid accumulation of Tol<sup>−</sup> mutants was observed in the absence of toluene and the presence of TCE. The percentage of toluene-negative cells in the culture might have increased by a higher growth rate of the mutants compared with wild-type cells, caused by a selective toxic effect of TCE on the wild type. This multiplication of toluene-negative cells can only be the result of cryptic growth on material released from lysed cells, because no additional carbon and energy source was added to the culture. This is possible, as was shown by Zambrano et al. (34), who described the takeover of stationary cultures of *Escherichia coli* by a mutant. If it is assumed that the mutants appeared because of selective growth of mutant cells that were present at the onset of toluene starvation, then it can be calculated from their observed growth rate ( $0.016 \text{ h}^{-1}$ ) that the amount of viable Tol<sup>−</sup> cells initially present was about  $6.7 \times 10^5 \text{ CFU ml}^{-1}$ . This is 0.3% of the total number of viable cells present. Replica plating indeed showed that Tol<sup>−</sup> mutants were present in such low numbers before toluene starvation (Fig. 2B).

Simultaneous removal of TCE and toluene is possible under nongrowth conditions in spite of the negative effects of TCE on the culture. TCE generates a large increase in maintenance energy demand of the cells and causes more rapid decay and the loss of the Tol<sup>+</sup> phenotype in the absence of toluene. This might result in a selective disadvantage towards toluene-degrading strains which do not convert TCE. This would be a real concern when cometabolic TCE-degrading strains are used in cleanup processes under nonsterile conditions. The risk of takeover of the TCE-degrading population by other toluene degraders seems rather high. Separation of degradation and growth, or the use of less common primary substrates, is under study.

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#### REFERENCES

1. Bailey, J. E., and D. F. Ollis. 1986. Biochemical engineering fundamentals, 2nd ed. McGraw-Hill Kogakusha Inc., Tokyo.
2. Bergmann, J. G., and J. Sanik. 1957. Determination of trace amounts of chlorine in naphtha. *Anal. Chem.* **29**:241–243.
3. Chesbro, W. 1988. The domains of slow bacterial growth. *Can. J. Microbiol.* **34**:427–435.
4. Chesbro, W., T. Evans, and R. Eifert. 1979. Very slow growth of *Escherichia coli*. *J. Bacteriol.* **139**:625–638.
5. Dolfing, J., A. J. van den Wijngaard, and D. B. Janssen. 1993. Microbial aspects of the removal of chlorinated hydrocarbons from air. *Biodegradation* **4**:261–282.
6. Ensley, B. D. 1991. Biochemical diversity of trichloroethylene metabolism. *Annu. Rev. Microbiol.* **45**:283–299.
7. Esener, A. A., J. A. Roels, and N. W. Kossen. 1981. Fed-batch culture: modeling and applications in the study of microbial energetics. *Biotechnol. Bioeng.* **22**:1851–1871.
8. Folsom, B. R., P. J. Chapman, and P. H. Pritchard. 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. *Appl. Environ. Microbiol.* **56**:1279–1285.
9. Fox, B. G., J. G. Borneman, L. P. Wackett, and J. D. Lipscomb. 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. *Biochemistry* **29**:6419–6427.
10. Henschler, D. 1985. Halogenated alkenes and alkynes, p. 317–347. *In* M. W.

- Anders (ed.), Bioactivation of foreign compounds. Academic Press, Inc., New York.
11. Henschler, D. 1994. Toxicity of chlorinated organic compounds: effect of the introduction of chlorine in organic molecules. *Angew. Chem. Int. Ed. Engl.* **33**:1920–1935.
  12. Janssen, D. B., A. Scheper, and B. Witholt. 1984. Biodegradation of 2-chloroethanol by pure bacterial cultures. *Prog. Ind. Microbiol.* **20**:169–178.
  13. Kado, C. I., and S.-T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. *J. Bacteriol.* **145**:1365–1373.
  14. Landa, A. S., E. M. Sipkema, J. Weijma, A. A. C. M. Beenackers, J. Dolfing, and D. B. Janssen. 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. *Appl. Environ. Microbiol.* **60**:3368–3374.
  15. Love, N. G., and C. P. L. Grady, Jr. 1995. Impact of growth on benzoate and *m*-toluate liquid media on culturability of *Pseudomonas putida* on benzoate and *m*-toluate plates. *Appl. Environ. Microbiol.* **61**:3142–3144.
  16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
  17. Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard. 1987. Biodegradation of trichloroethylene and involvement of an aromatic pathway. *Appl. Environ. Microbiol.* **53**:949–954.
  18. Nelson, M. J. K., S. O. Montgomery, O. J. O'Neill, and P. H. Pritchard. 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. *Appl. Environ. Microbiol.* **52**:383–384.
  19. Newman, L. M., and L. P. Wackett. 1995. Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. *Biochemistry* **34**:14066–14076.
  20. Oldenhuis, R., J. Y. Oedzes, J. J. van der Waarde, and D. B. Janssen. 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. *Appl. Environ. Microbiol.* **57**:7–14.
  21. Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt. 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. *Appl. Environ. Microbiol.* **55**:2819–2826.
  22. Pirt, S. J. 1965. The maintenance energy of bacteria in growing cultures. *Proc. R. Soc. Lond. B* **163**:224–231.
  23. Rohde, C. 1995. Technical information sheet no. 12. Plasmid isolation from bacteria: some fast procedures. *World J. Microbiol. Biotechnol.* **11**:367–369.
  24. Shields, M. S., S. O. Montgomery, P. J. Chapman, S. M. Cuskey, and P. H. Pritchard. 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. *Appl. Environ. Microbiol.* **55**:1624–1629.
  25. Shields, M. S., S. O. Montgomery, S. M. Cuskey, P. J. Chapman, and P. H. Pritchard. 1991. Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. *Appl. Environ. Microbiol.* **57**:1935–1941.
  26. Shields, M. S., M. J. Reagin, R. R. Gerger, R. Campbell, and C. Somerville. 1995. TOM, a new aromatic degradative plasmid from *Burkholderia (Pseudomonas) cepacia* G4. *Appl. Environ. Microbiol.* **61**:1352–1356.
  27. Smith, M. R. 1990. The biodegradation of aromatic hydrocarbons by bacteria. *Biodegradation* **1**:191–206.
  28. Solomon, B. O., C. Posten, M. P. F. Harder, V. Hecht, and W.-D. Deckwer. 1994. Energetics of *Pseudomonas cepacia* G4 growth in a chemostat with phenol limitation. *J. Chem. Technol. Biotechnol.* **60**:275–282.
  29. Tsien, H.-C., G. A. Brusseau, R. S. Hanson, and L. P. Wackett. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. *Appl. Environ. Microbiol.* **55**:3155–3161.
  30. van Verseveld, H. W., M. Braster, F. C. Booger, B. Chance, and A. H. Stouthamer. 1983. Energetic aspects of growth of *Paracoccus denitrificans*: oxygen-limitation and shift from anaerobic nitrate-limitation to aerobic succinate limitation. *Arch. Microbiol.* **135**:229–236.
  31. van Verseveld, H. W., J. A. de Hollander, J. Frankena, M. Braster, F. J. Leeuwerik, and A. H. Stouthamer. 1986. Modeling of microbial substrate conversion, growth and product formation in a recycling fermentor. *Antonie van Leeuwenhoek* **52**:325–342.
  32. Wackett, L. P. 1991. Dehalogenation of organohalide pollutants by bacterial enzymes and coenzymes, p. 191–200. *In* J. W. Kelly and T. O. Baldwin (ed.), *Applications of enzyme biotechnology*. Plenum Press, New York.
  33. Wackett, L. P., and S. R. Householder. 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. *Appl. Environ. Microbiol.* **55**:2723–2725.
  34. Zambrano, M. M., D. A. Siegle, M. Almiron, A. Tormo, and R. Kolter. 1993. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* **259**:1757–1759.