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Effect of carbon starvation on toluene degradation activity by toluene monooxygenase-expressing bacteria

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Abstract

Subsurface bacteria commonly exist in a starvation state with only periodic exposure to utilizable sources of carbon and energy. In this study, the effect of carbon starvation on aerobic toluene degradation was quantitatively evaluated with a selection of bacteria representing all the known toluene oxygenase enzyme pathways. For all the investigated strains, the rate of toluene biodegradation decreased exponentially with starvation time. First-order deactivation rate constants for TMO-expressing bacteria were approximately an order of magnitude greater than those for other oxygenase-expressing bacteria. When growth conditions (the type of growth substrate and the type and concentration of toluene oxygenase inducer) were varied in the cultures prior to the deactivation experiments, the rate of deactivation was not significantly affected, suggesting that the rate of deactivation is independent of previous substrate/inducer conditions. Because TMO-expressing bacteria are known to efficiently detoxify TCE in subsurface environments, these findings have significant implications for *in situ* TCE bioremediation, specifically for environments experiencing variable growth-substrate exposure conditions.

Abbreviations: AMO – ammonia monooxygenase; BM – basal salt medium; CFU – colony forming unit; MMO – methane monooxygenase; TCE – trichloroethylene; TDO – toluene dioxygenase; TMO – toluene monooxygenase; T4MO – toluene-4-monooxygenase; TNA – tryptone nutrient agar

Introduction

Trichloroethylene (TCE), a widespread contaminant of soil and groundwater, can be co-oxidized in aerobic environments by a variety of aromatic and aliphatic bacterial oxygenases (Arciero et al. 1989; Ensign et al. 1992; Folsom et al. 1990; Fox et al. 1990; Heald & Jenkins 1994; Lontoh & Semrau 1998; Malachowsky et al. 1994; Zylstra et al. 1989). Utilization of oxygenase-expressing bacteria for *in situ* biodegradation of TCE, therefore, is receiving attention as a potential and promising remediation strategy. Toluene monooxygenase (TMO)-expressing bacteria are of specific interest because they are relatively tolerant to TCE-mediated toxicity (Mars et al. 1996; Park et al. 2002). Furthermore, at a site undergoing toluene addition to stimulate TCE co-oxidation,

TMO-expressing bacteria were identified as a major aromatic-degrading population (Hopkins & McCarty 1995; McCarty et al. 1998).

Environmental stress conditions can significantly affect cellular physiology, such as rates of biodegradation. For subsurface bacteria, understanding the effects of carbon starvation on rates of biodegradation is of particular interest because the subsurface environment is commonly carbon-limited (Ghiorse & Wilson 1988; Morita 1993). Several investigations with oxygenase-expressing bacteria have explored the effects of carbon starvation on biodegradation activity. These investigations include experiments with bacteria that express ammonia monooxygenase (AMO) (Jones & Morita 1985), methane monooxygenase (MMO) (Alvarez-Cohen & McCarty 1991; Henry & Grbac-Galic 1991; Roslev & King 1994), toluene dioxygenase (TDO) (Costura & Alvarez 2000; Jenkins & Heald 1996), and toluene-4-monooxygenase (T4MO) (Park et al. 2001). Experiments revealed that the rate of primary substrate biodegradation is reduced exponentially with increasing carbon starvation time. Interestingly, when first-order decay models were applied to experimental data, the first-order rate constant (deactivation rate) for the T4MO-expresing *R. pickettii* PKO1 bacterium (4.68 d^{-1}) was more than an order of magnitude greater than deactivation rates observed for the other oxygenase-expressing bacteria (< 0.2–0.36 d^{-1}) (Costura & Alvarez 2000; Jenkins & Heald 1996; Jones & Morita 1985; Park 2001; Roslev & King 1994). Such a wide range of deactivation rates suggests that deactivation may have a significant influence on the effectiveness of bioremediation processes and the accuracy of *in situ* biodegradation rate predictions (Lang et al. 1997; Park et al. 2001). However, the dependence of deactivation rates on biological and environmental factors is currently unclear, complicating the ability to incorporate deactivation information in prediction and design methodologies.

The primary objective of this work was to examine whether rapid deactivation of biodegradation activity in response to carbon starvation is a general characteristic of TMO-expressing bacteria. To explore this question, the effect of carbon starvation on toluene degradation activity was quantified for a selection of bacteria representing all the known toluene oxygenase enzyme pathways. The bacterial strains investigated in this study and their corresponding toluene oxygenase pathways are *Burkholderia cepacia* G4 (Figure 1, pathway A), *Ralstonia pickettii* PKO1 (Figure 1, pathway B), *Pseudomonas mendocina* KR1 (Figure 1, pathway C), *Pseudomonas putida* mt-2 (Figure 1, pathway D), and *Pseudomonas putida* F1 (Figure 1, pathway E). With the exception of the toluene methyl monooxygenase pathway (Figure 1, pathway D), all the toluene biotransformation pathways can co-oxidize TCE (Malachowsky et al. 1994; Leahy et al. 1996; Shields et al. 1989; Zylstra et al. 1989). Because subsurface bacteria are commonly exposed to highly variable growth/induction conditions (McCarty et al. 1998; Vroblesky & Chapelle 1994), the dependence of deactivation rates on prior substrate/inducer exposure conditions was also investigated.

Materials and methods

Bacterial strains and growth conditions

The following bacterial strains were grown in pure culture: *B. cepacia* G4, *B. cepacia* G4-$PR1_{31}$, *P. mendocina* KR1, *P. putida* F1, *P. putida* mt-2, and *R. pickettii* PKO1 (see Table 1 for relevant strain characteristics). Cells previously stored at −70 °C were serially diluted and plated on tryptone nutrient agar (TNA) solid media (Olsen & Hansen 1976). After incubation for 3 days at 30 °C, 10 colonies were transferred to a 2-l culture flask and resuspended in 200 ml of pre-oxygenated basal salts medium (BM) (Leahy et al. 1996). Uninduced cells were grown by amending culture flasks with 1000 mg sodium DL-lactate l^{-1} followed by incubation for 18 h at 30 °C. A 1:10 liquid to headspace ratio and shaking maintained aerobic conditions during cellular growth. Our preliminary examination with a similar experimental set-up ensured aerobic conditions (aqueous O_2 concentrations > 2.5 mg l^{-1}) throughout the growth cycle. After incubation, the A_{425} (optical density at 425 nm) of late-exponential-phase cell cultures was typically between 0.8 and 1.0.

Toluene-induced cells were grown by amending culture flasks with toluene as the only growth substrate or with toluene plus 1000 mg sodium DL-lactate l^{-1} and incubating for 18–72 h at 30 °C. Toluene was supplied by suspending a 1.8-ml glass

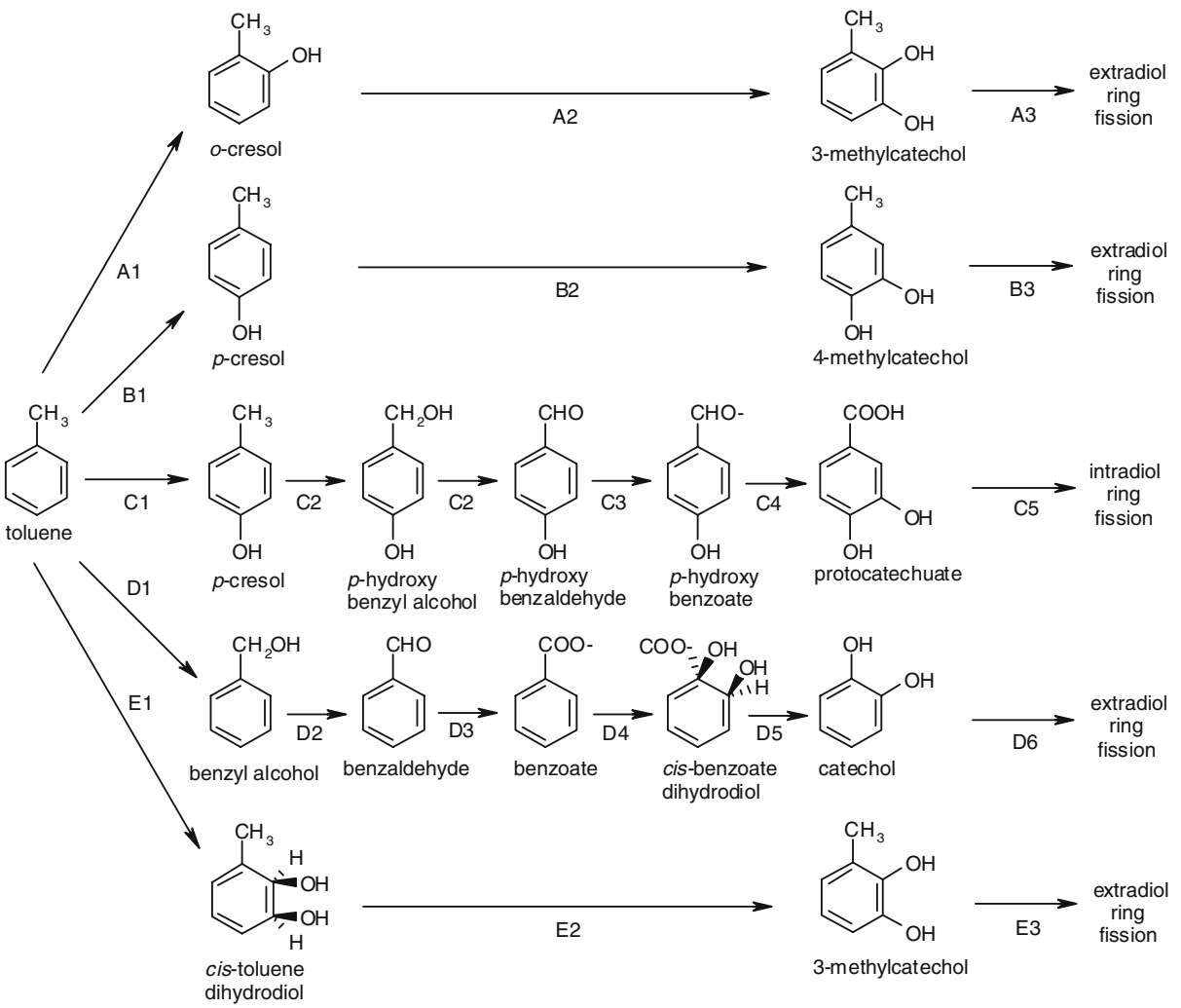

Figure 1. Toluene oxygenase-mediated pathways for the aerobic biotransformation of toluene. Toluene-2-monooxygenase pathway (Pathway A): (A1) toluene-2-monooxygenase, (A2) *o*-cresol hydroxylase, and (A3) 3-methylcatechol-2,3-dioxygenase. Toluene-4-monooxygenase pathway followed by ring cleavage of 4-methyl catechol (Pathway B): (B1) toluene-4-monooxygenase, (B2) *p*-cresol hydroxylase, and (B3) 4-methylcatechol-3,4-dioxygenase. Toluene-4-monooxygenasae pathway followed by ring cleavage of protocatechuate (Pathway C): (C1) toluene-4-monooxygenase, (C2) *p*-cresol methylhydroxylase, (C3) *p*-hydroxybenzaldehyde dehydrogenase, (C4) *p*-hydroxybenzoate hydroxylase, and (C5) protocatechuate-3,4-dioxygenase. Toluene methyl monooxygenase pathway (Pathway D): (D1) methyl monooxygenase, (D2) benzyl alcohol dehydrogenase, (D3) benzaldehyde dehydrogenase, (D4) benzoate-1,2-dioxygenase, (D5) dihydroxycyclohexadiene carboxylate dehydrogenase, and (D6) catechol-2,3-oxygenase. Toluene dioxygenase pathway (Pathway E): (E1) toluene-2,3-dioxygenase, (E2) toluene dihydrodiol dehydrogenase, and (E3) 3-methylcatechol-2,3-dioxygenase.

vial containing 0.5 ml of liquid toluene from the culture flask stopper. For similar experimental setups, the resulting aqueous toluene concentration in the culture flask was approximately 230 mg l^{-1}, ensuring enzymatic induction (Leahy & Olsen 1997).

TCE-induced cells were grown by transferring 1 ml of lactate-grown uninduced cell culture (A_{425} of 0.8–1.0) to a 160-ml serum bottle containing 9 ml of preoxygenated BM, 1000 mg sodium DL-lactate l^{-1}, and either 50, 110, or 150 mg TCE l^{-1}. The TCE stock was prepared in *N′N′*-dimethylformamide (900 g TCE per liter of *N′N′*-dimethylformamide) and added directly to the aqueous phase. The TCE concentration in each serum bottle was reported based upon the initial

Table 1. Characteristics of toluene utilizing bacteria employed in this study and measured first-order deactivation rates

Bacterial strain	Toluene metabolism pathway	Subclass of *Proteobacteria*	Location of toluene oxygenase operon	Maximum specific toluene growth rate (h^{-1})	Deactivation rate constant, k_D (d^{-1})	95% Confidence interval (d^{-1})	Correlation coefficient of first-order model
B. cepacia G4[a]	2-Monooxygenase[b] (aryl oxidation)	Beta	Plasmid[c]	0.22[d]	2.19	1.82, 2.56	0.962
R. pickettii PKO1[a]	4-Monooxygenase followed by ring cleavage of 4-methyl catechol[e] (aryl oxidation)	Beta	Chromosome[f]	0.16[d]	4.68	3.77, 5.59	0.998
P. mendocina KR1[g]	4-Monooxygenase followed by ring cleavage of protocatechuate[h] (aryl and alkyl oxidation)	Gamma	Chromosome[i]	0.45[j]	4.04	3.06, 5.02	0.948
P. putida mt-2[g]	Methyl monooxygenase[k] (alkyl oxidation)	Gamma	Plasmid[k]	0.40[j]	3.82	3.59, 4.05	0.996
P. putida F1[g]	2,3-Dioxygenase[l] (aryl oxidation)	Gamma	Chromosome[m]	0.38[j]	0.39	0.28, 0.50	0.977

[a]Cells grown with saturating toluene vapors and 1000 mg sodium DL-lactate l^{-1}; [b]Shields et al. (1989); [c]Shields et al. (1995); [d]Massol-Deyá et al. (1997); [e]Fishman et al. (2004); [f]Olsen et al. (1994); [g]Cells grown with saturating toluene vapors; [h]Whited & Gibson (1991); [i]Wright & Olsen (1994); [j]Duetz et al. (1994); [k]Worsey & Williams (1975); [l]Gibson et al. (1970); [m]Zylstra et al. (1988).

concentration in the aqueous phase. After the addition of TCE, the serum bottles were sealed with Teflon-lined butyl septa, vigorously shaken for 30 s, and incubated for 18 h at 30 °C.

Starvation cultures were obtained by harvesting toluene- and TCE-induced cells by centrifugation, washing cell pellets with 40 mM phosphate buffer (pH 6.7–7.0), and resuspending cells in 200 ml fresh BM to an optical density of 0.1. Starvation flasks were maintained at room temperature (23–27 °C) with continuous mixing. In the case of the starvation experiment with previously TCE-induced cells, TCE was added at concentrations similar to those used in the previous TCE induction conditions in order to examine the effect of carbon starvation *per se* in the presence of inducer.

Toluene degradation kinetics

Rates of toluene degradation were measured in 15-ml bottles. Each bottle was completely filled with culture or with deionized water to serve as a no-cell control for abiotic losses. Bottles were sealed with Teflon-lined butyl septa, amended with liquid toluene to obtain an aqueous concentration of 10 mg toluene l^{-1}, and vigorously and continuously mixed. Four aqueous 0.5-ml samples were taken at periodic time intervals after toluene addition using a 1-ml glass syringe. Sampling intervals for specific bacterial strains were as follows: *P. putida* F1, 2 min; *P. putida* mt-2, 2 min; *P. mendocina* KR1, 4 min; *B. cepacia* G4, 4 min; *B. cepacia* G4-PR1$_{31}$, 4 min; *R. pickettii* PKO1, 15 min. After sample collection, samples were immediately quenched in 1-volume methanol and stored at 4 °C prior to quantitative analysis. Toluene concentrations for each time point were used to derive linear relationships by regression analysis, providing a quantification of the rate of toluene disappearance. To quantify rate data, the average rate loss of toluene from duplicate abiotic controls was subtracted from the average rate loss of toluene in duplicate biotic experiments. Rates were quantified over the time for disappearance of 30–40% of the initial mass of toluene from the biotic experiments. Less than 5% of the total initial mass of toluene was lost in abiotic controls over the same period of time. This also ensured that there would be insignificant effect of toluene partitioning on the measurement of aqueous

toluene concentrations. Viable biomass-normalized toluene degradation rates were obtained by normalizing toluene degradation rates by the number of colony forming units per reaction volume.

Deactivation rates

Toluene degradation rates and their corresponding starvation times were used to derive first-order relationships using the following equation:

$$V(t_{cs}) = V_o \cdot \exp[-k_D \cdot t_{cs}]$$

where t_{cs}, carbon starvation period; $V(t_{cs})$, specific (biomass-normalized) rate of degradation of toluene as a function of the carbon starvation period t_{cs}; V_o, specific rate of degradation of toluene by non-starved and induced cells; and k_D, first order deactivation rate constant. The deactivation rate, 95% confidence intervals (CIs), and correlation coefficient were obtained by non-linear regression analysis using the SPSS Regression Models software package (SPSS Inc.). Deactivation rates with overlapping 95% CIs were considered statistically similar while deactivation rates with non-overlapping 95% CIs were considered statistically different.

Toluene culturability assays

A modification of the toluene plate count method described by Mars and co-workers (1996) was used to quantify the fraction of carbon-starved cells retaining their ability to grow on toluene. Cell suspensions were starved for 4 days, transferred to TNA agar plates, and incubated for 3 days at 30 °C. Colonies were subsequently transferred to solid BM medium and continuously supplied with toluene vapors by adding 5 μl of liquid toluene to a filter paper disk located in the cover of the plate. The toluene-supplied plates were stored in airtight glass containers for 10 days and the number of resulting colonies was quantified.

Analytical methods

Toluene was analyzed by reverse-phase high-performance liquid chromatography (HPLC) with a Hewlett-Packard 1090 series II system, a Hypersil $5C_{18}$ column, and a UV detection system (210 nm). The carrier solvent solution was 70% acetonitrile and 30% deionized water with a flow rate of 0.35 ml min^{-1}. Detection limits for toluene and TCE were 10 and 1 $\mu g\ l^{-1}$, respectively.

Viable biomass was quantified by a colony plate-count method. Sample cultures were serially diluted and cells were grown on TNA agar plates for 3 days at 30 °C. A single viable cell was assumed to form from one colony (1 CFU). Triplicate dilution series and plate counts were performed for each experimental sample.

Results

Rapid deactivation in TMO-expressing bacteria

All the investigated TMO-expressing bacteria deactivate significantly faster than the TDO-expressing bacterium (Table 1). The deactivation rates do not correlate with other biological characteristics, including phylogenetic classification, maximum specific toluene growth rates, and the location of the toluene oxygenase operon (plasmid or chromosome).

Toluene culturability after carbon starvation

Deactivation may result from the loss of the functional TMO operon, such as by plasmid curing (Duetz & van Andel 1991; Williams et al. 1988) or by a loss-of-function mutation. The contribution of this type of genetic change towards the observed deactivation responses was assessed by performing a toluene culturability assay. With the exception of *P. putida* mt-2, 100% of the cells retained their ability to degrade toluene after four days of carbon starvation (Table 2). Even for *P. putida* mt-2, whose toluene oxygenase operon is located on a mobile plasmid (Williams et al. 1988), the number of cells losing their ability to grow on toluene could not account for the observed deactivation behavior.

Effect of previous growth/induction conditions on deactivation

The effect of previous substrate exposure conditions was examined by growing *B. cepacia* G4 with either (i) toluene only or (ii) toluene plus lactate. The co-presence of lactate during growth

Table 2. Toluene culturability assays

Bacterial strain	Number of colonies on TNA plates after carbon starvation[a]	Number of colonies on toluene-BM plates after carbon starvation[b]
R. pickettii PKO1	100	100
P. mendocina KR1	100	100
P. putida mt-2	100	91
B. cepacia G4	100	100
P. putida F1	100	100

[a]After incubation for 3 days on TNA plates, cells were transferred to toluene-BM plates and incubated for 10 days.
[b]Cells were starved for 4 days prior to growth on TNA plates.

conditions significantly reduced the initial rate of toluene degradation in non-starved cells (time = 0 in Figure 2). However, the deactivation rates for the two growth conditions were statistically similar (Table 3), providing evidence that the rate of deactivation is independent of prior substrate exposure conditions.

The effect of previous induction conditions on the rate of deactivation was tested by exposing lactate-grown *R. pickettii* PKO1 cells to various concentrations of TCE during both growth and starvation conditions (Table 3). Because TCE induces T4MO activity but is not a metabolic substrate of the organism (Park et al. 2002), these growth and starvation conditions allowed the effects of carbon starvation and the absence of an inducer to be decoupled. In addition, the selected range of TCE concentrations allowed the effect of TCE toxicity on the deactivation rate to be examined, where the highest concentration of TCE (150 mg l^{-1}) is toxic to *R. pickettii* PKO1 and the lower concentrations of TCE (110 and 55 mg l^{-1}) are less toxic (Park et al. 2002). Results show that the deactivation rates for cells exposed to different TCE concentrations are all statistically similar, although the deactivation rate for the toxic concentration of 150 mg TCE l^{-1} was smaller than the deactivation rates for the less toxic concentrations of 55 and 110 mg TCE l^{-1} (Figure 3, Table 3). These results demonstrate that the rate of deactivation is not significantly affected by the concentration of the inducer. In addition, the deactivation rates for the less toxic concentrations of 55 and 110 mg TCE l^{-1} were not significantly different from the deactivation rate for cells previously grown and induced with toluene (Table 3), suggesting that the deactivation rate is independent of the type of inducer (TCE or toluene).

Furthermore, the insignificant differences between deactivation rates for cells starved in the presence (55 and 110 mg TCE l^{-1}) and absence (toluene starvation) of an inducer indicates that

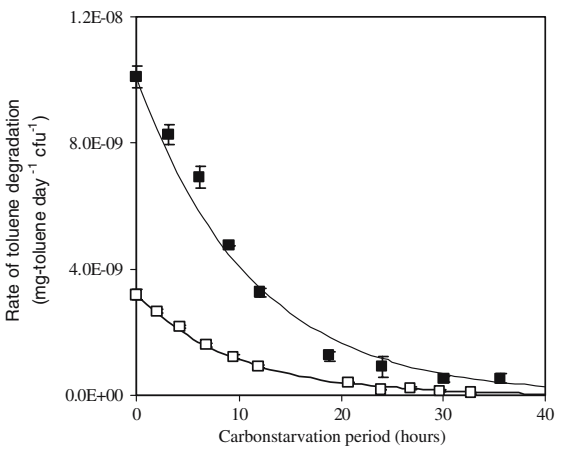

Figure 2. Deactivation of toluene degradation activity in *B. cepacia* G4 cells grown with (■) saturating toluene vapors or (□) saturating toluene vapors and 1000 mg sodium DL-lactate l^{-1}. Measurements are averages of duplicate experiments and error bars represent one standard deviation. Solid lines are the first-order model predictions obtained by non-linear regression of the experimental data.

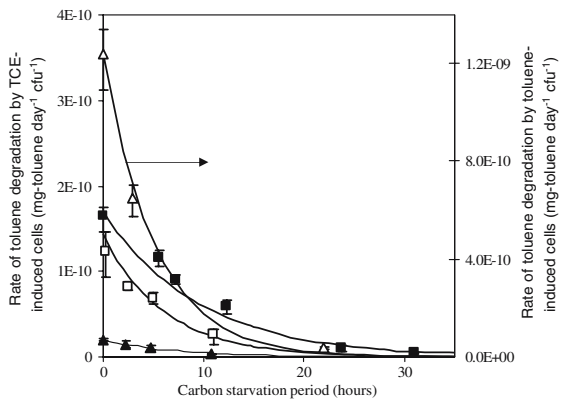

Figure 3. Deactivation of toluene degradation activity in *R. pickettii* PKO1 cells grown with 1000 mg sodium DL-lactate l^{-1} and (▲) 55 ppm TCE, (□) 110 ppm TCE, (■) 150 ppm TCE, or (Δ) saturating toluene vapors. TCE concentrations present during growth conditions were maintained throughout starvation conditions. Measurements are averages of duplicate experiments and error bars represent one standard deviation. Solid lines are the first-order model predictions obtained by non-linear regression of the experimental data.

Table 3. First-order deactivation rates and growth/induction conditions

Bacterial strain	Type of regulation	Growth conditions[a]	Starvation conditions	Deactivation rate constant k_D (d^{-1})	95% Confidence interval (d^{-1})	Correlation coefficient of first-order model
B. cepacia G4	Inducible[b]	Toluene		2.46	2.22, 2.70	0.962
		Toluene + Lactate		2.19	1.82, 2.56	0.993
B. cepacia G4-PR131	Constitutive[c]	Toluene + Lactate		1.48	1.03, 1.93	0.979
R. pickettii PKO1	Inducible[d]	Toluene + Lactate		4.68	3.77, 5.59	0.998
		150 mg TCE l^{-1} + Lactate	55 mg TCE l^{-1}	3.53	2.49, 4.57	0.992
		110 mg TCE l^{-1} + Lactate	110 mg TCE l^{-1}	3.91	2.40, 5.42	0.969
		55 mg TCE l^{-1} + Lactate	150 mg TCE l^{-1}	2.51	1.93, 3.09	0.986

[a]Lactate was supplied at a concentration of 1000 mg sodium DL-lactate l^{-1}, toluene was supplied with saturating toluene vapors; [b]Shields et al. (1989); [c]Shields & Reagin (1992); [d]Olsen et al. (1994).

deactivation results from carbon starvation and not from the absence of a TMO inducer. This was further supported by comparing deactivations for the induction-dependent *B. cepacia* G4 and it's constitutively expressing mutant *B. cepacia* G4-$PR1_{31}$. A significant deactivation was also observed for the constitutively expressing variant although the rate of deactivation was lower than that for the induction-dependent G4 strain.

Discussion

The deactivation rates for all the investigated TMO-expressing bacteria (2.19–4.68 d^{-1}) were significantly larger than the measured and previously reported deactivation rates for TDO-expressing bacteria (0.39 d^{-1} [this study]; 0.36 d^{-1} [Costura & Alvarez 2000]; 0.30 d^{-1} [Jenkins & Heald 1996]). Although minor differences in experimental design between this study and the referenced studies exist, the deactivation rates for *P. putida* F1 measured in this study and by Costura & Alvarez (2000) were similar, validating the previous comparison. The deactivation rates for the TMO-expressing bacteria reported here were also significantly larger than those reported for other monooxygenase-expressing bacteria (0.21 d^{-1} for AMO [Jones & Morita 1985]; less than 0.2 d^{-1} for MMO [Roslev & King 1994]). Together, these observations provide substantial evidence supporting the hypothesis that rapid deactivation is a general characteristic of TMO-expressing bacteria. Further support for this observation is provided by the lack of a correlation between deactivation rates and other organism-specific characteristics, including phylogenetic classification, maximum toluene growth rates, the location of the toluene oxygenase operon (chromosome or plasmid), or the type of regulation of the oxygenase operon (constitutive or inducible).

An analysis of the biochemistry of toluene oxygenase pathways can provide insight into the potential mechanisms controlling the deactivation of *in vivo* toluene oxygenase activity. TMO pathways require a net consumption of NADH for the toluene biotransformation steps preceding ring cleavage (Wackett & Hershberger 2001). Based upon these observations, it is reasonable to hypothesize that the fast deactivation of TMO-expressing bacteria may be related to its higher demand for NADH. This hypothesis implies that the NADH demand for the initial catabolic step is greater than the NADH demand for cellular maintenance and respiration. Further investigations that quantify NADH levels as a function of starvation time would be needed to explore the validity of this hypothesis.

An additional and important finding of this study is that the rate of deactivation in TMO-expressing bacteria is independent of previous substrate/inducer exposure conditions, including the co-presence of a more readily degradable substrate and the type, concentration, and existence of an inducer during starvation conditions. In another study with *P. putida* F1, Costura & Alvarez (2000) demonstrated that the deactivation rate was independent of previous respiration

conditions (e.g. oxygen concentration). Taken together with the findings reported here, this general independence of deactivation rates on previous culture conditions suggests that the extent of deactivation for a particular organism depends only on the length of the carbon starvation period.

The results presented here have the potential to significantly improve bioremediation design and prediction methodologies. Biostimulation processes involving the addition of a primary growth substrate commonly utilize pulse injections to improve mixing and spatial distribution (McCarty et al. 1998). The addition of pulse injections, however, can result in a condition where organisms experience highly fluctuating substrate concentrations and possible periods of carbon and energy limitations (Lang et al. 1997; McCarty et al. 1998; Park et al. 2001). Accounting for starvation effects, therefore, would be useful for designing the length of the substrate pulse such that deactivation of biodegradation activity is minimized. Additionally, accounting for the effects of carbon starvation would improve the accuracy of quantitative models describing biodegradation in complex natural systems. These models would, in turn, provide more accurate predictions of bioremediation rates and capacity.

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