Biodegradation kinetics of toluene, *m*-xylene, *p*-xylene and their intermediates through the upper TOL pathway in *Pseudomonas putida* (pWW0)

Wouter A. Duetz,¹ Bea Wind,² Johan G. van Andel,² Michael R. Barnes,³ Peter A. Williams³ and Michiel Rutgers²

Author for correspondence: Wouter A. Duetz. Tel: +41 1 6333811. Fax: +41 1 6331051.
e-mail: duetz@biotech.biol.ethz.ch

¹ Institut für Biotechnologie, ETH Hönggerberg, HPT, CH 8093 Zürich, Switzerland

² Laboratory of Ecotoxicology, National Institute of Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands

³ School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, UK

***Pseudomonas putida* mt-2, harbouring TOL plasmid pWW0, is capable of degrading toluene and a range of di- and tri-alkylbenzenes. In this study, chemostat-grown cells ($D = 0.05 \text{ h}^{-1}$, toluene or *m*-xylene limitation) of this strain were used to assess the kinetics of the degradation of toluene, *m*-xylene, *p*-xylene, and a number of their pathway intermediates. The conversion kinetics for the three hydrocarbons showed significant differences: the maximal conversion rates were rather similar [$11\text{--}14 \text{ mmol h}^{-1} (\text{g dry wt})^{-1}$] but the specific affinity (the slope of the v vs s curve near the origin) of the cells for toluene [$1300 \text{ l (g dry wt)}^{-1} \text{ h}^{-1}$] was only 5% and 14% of those found for *m*-xylene and *p*-xylene, respectively. Consumption kinetics of mixtures of the hydrocarbons confirmed that xylenes are strongly preferred over toluene at low substrate concentrations. The maximum flux rates of pathway intermediates through the various steps of the TOL pathway as far as ring cleavage were also determined. Supply of 0.5 mM 3-methylbenzyl alcohol or 3-methylbenzaldehyde to fully induced cells led to the transient accumulation of 3-methylbenzoate. Accumulation of the corresponding carboxylic acid (benzoate) was also observed after pulses of benzyl alcohol and benzaldehyde, which are intermediates in toluene catabolism. Analysis of consumption and accumulation rates for the various intermediates showed that the maximal rates at which the initial monooxygenation step and the conversion of the carboxylic acids by toluate 1,2-dioxygenase may occur are two- to threefold lower than those measured for the two intermediate dehydrogenation steps.**

Keywords: BTEX, flux study, specific affinity, aromatic hydrocarbons, Michaelis–Menten kinetics

INTRODUCTION

Pseudomonas putida mt-2 was isolated in Japan from a *m*-toluate enrichment culture in 1959 by the group of Hayaishi. Fifteen years passed before the same strain was found to be capable of degrading toluene, *m*-xylene, *p*-xylene and a number of other di- and tri-alkyl-substituted benzenes (Worsey & Williams, 1975; Kunz & Chapman, 1981; Assinder & Williams, 1990). The observation that the toluene catabolic genes are located

on a large catabolic plasmid (designated TOL plasmid pWW0), and the apparent importance of the so-called TOL pathway in the natural attenuation of the aromatic fraction of spilled oil-products, caused *P. putida* mt-2 to become one of the most intensively studied bacterial strains of the last two decades (reviewed by Assinder & Williams, 1990; Marqués *et al.*, 1994; Ramos *et al.*, 1997).

In soil samples taken from various petrol-contaminated sites, 30–50% of the toluene-degrading bacterial population harbours a TOL pathway (as determined by oxygen-uptake experiments; unpublished results). In environments contaminated with oil-products, toluene and the xylenes are often present together (Chapelle,

Abbreviations: a_A^0 , specific affinity; D , dilution rate; K_m , Michaelis–Menten constant; K_s , Monod constant; K_T , half-saturation constant for uptake; V_{max} , maximal conversion rate.

1993). However, it is unknown to what extent toluene and to what extent di- and tri-alkylsubstituted benzenes contribute to the total flux through the TOL pathway under such mixed-substrate conditions. To investigate this question we compared the degradation kinetics ($V_{\max}$, K_T , specific affinity) for the three substrates of the TOL pathway that are quantitatively the most important in oil products (toluene, *m*-xylene and *p*-xylene: see Chapelle, 1993). In addition, we studied the (maximum) flux of intermediates of these compounds through the first steps of the TOL pathway.

METHODS

Bacterial strain. *P. putida* mt-2 (ATCC 33015) harbouring the TOL plasmid pWW0, hereafter referred to as *P. putida* (pWW0), was stored in a 20% (v/v) glycerol culture at -70°C .

Culture medium. The composition of the mineral-salts medium used for cultivation of the strain under carbon limitation in chemostat culture was described previously (Duetz *et al.*, 1991).

Chemostat culture. The fermenters used were custom-made, shaped like an Erlenmeyer flask, and had a working volume of 105 ± 5 ml. Details of the fermenter can be found in Duetz *et al.* (1996). The temperature was maintained at $28 \pm 1^\circ\text{C}$. *m*-Xylene or toluene were supplied by mixing the main airflow (6 l h^{-1}) with air saturated (at 0°C) with *m*-xylene (300 ml h^{-1}) or toluene (70 ml h^{-1}), corresponding to an inflow of 24.0 and 25.2 $\mu\text{mol h}^{-1}$ respectively. The operational dilution rate (D) was 0.05 h^{-1} .

Analysis of *m*-xylene and toluene in the gas phase by GC. The percentage of *m*-xylene and toluene that was converted in the fermenter was calculated from the peak areas resulting from the injection of 250 μl samples of the incoming air and the headspace of the fermenter in a Hewlett Packard type 5890 gas chromatograph equipped with an HP101 methylsilicone capillary column (25 m), a split injector (1:7) (both at 100°C) and a flame ionization detector. A gas-tight glass syringe (SGE, Melbourne, Australia) with a stainless steel plunger (with no Teflon parts) was used for injection. The retention times for toluene, *m*-xylene and *p*-xylene were 1.4, 1.7 and 1.7 min, respectively.

Analysis of pathway intermediates. Samples were taken from a fermenter and filtered through a $0.22\text{ }\mu\text{m}$ filter within 5 s. For analysis of the benzyl alcohols, benzaldehydes, benzoates, dihydrodiols and catechols, samples of 1 μl were injected into an HPLC apparatus equipped with a variable-wavelength detector (Hewlett Packard 1050 series) set at 195 nm and a 10 cm Hypersil C18 column (Chrompack). The solvent was acetonitrile/5 mM H_2SO_4 (86:14, v/v) run at a flow rate of 0.4 ml min^{-1} .

Biomass concentration. The OD_{540} of chemostat cultures was measured regularly. As described in a previous study (Duetz *et al.*, 1996) no significant influence of the growth condition on the relationship between the data for the dry weight and OD_{540} values was found; under all conditions tested, an OD_{540} of 1.0 corresponded to a dry weight of $380 \pm 10\text{ mg l}^{-1}$, which was used to estimate the dry weight from the OD_{540} data.

Determination of the conversion kinetics of toluene, *m*- and *p*-xylene. The substrate-depletion method as described by Robinson & Characklis (1984) was used to determine the degradation kinetics ($V_{\max}$, K_T , specific affinity) of the volatile substrates of the TOL pathway. Cells were harvested from a

chemostat culture, diluted and transferred to a headspace vial. The suspension was stirred vigorously and supplied with toluene, *m*-xylene or *p*-xylene through the gas phase. The concentration was monitored by manual injection of 250 μl of the headspace into a gas chromatograph (as described above) every 2–4 min, using a gas-tight syringe. Further details of the technical and mathematical procedures can be found in Duetz *et al.* (1997).

Determination of the flux of intermediates through the TOL pathway. A 5 ml sample of cells of *P. putida* (pWW0) was harvested from a *m*-xylene-limited chemostat culture ($D = 0.05\text{ h}^{-1}$) and mixed with an equal volume of sterile mineral medium in a thermostatically controlled (28°C) 50 ml Erlenmeyer flask. Subsequently, the cell suspension was supplied with 400–500 μM benzyl alcohol, 3-methylbenzyl alcohol, benzaldehyde or 3-methylbenzaldehyde. At 5 min intervals, 1 ml samples were filtered through a $0.22\text{ }\mu\text{m}$ filter and stored at 0°C for later analysis by HPLC as described above. The specific fluxes of the compounds through the various steps of the TOL pathway were calculated from measures of changes in substrate concentrations with time.

RESULTS

P. putida (pWW0) was grown in chemostat culture at a dilution rate of 0.05 h^{-1} with either toluene or *m*-xylene as a growth-limiting carbon source. The cell densities under steady-state conditions were between 0.19 and 0.23 g dry wt l^{-1} . The actual fluxes of toluene and *m*-xylene in the chemostat cultures were 1.5 and 1.3 mmol h^{-1} (g dry wt) $^{-1}$, respectively. Expressed in mass units, the fluxes for both compounds were identical: 0.14 g h^{-1} (g dry wt) $^{-1}$. No metabolites were detected in the culture supernatants.

Kinetics of toluene, *m*-xylene and *p*-xylene utilization

The substrate-depletion method of Robinson & Characklis (1984) using headspace sampling was used to prepare v vs s curves for the three hydrocarbons. The applicability of this method is dependent on the continuous equilibrium between the gas phase and the liquid phase. The latter was proven by supplying the liquid phase with 20 μl phosphoric acid in the middle of a run (stopping the activity of the cells), resulting in the instantaneous halting of the decrease in the *m*-xylene concentration in the gas phase. v vs s curves for *m*-xylene and *p*-xylene utilization were made using cells harvested from the *m*-xylene-limited chemostat culture (Fig. 1). The v vs s curve for toluene utilization as presented in Fig. 1 was derived in an identical way using cells harvested from the toluene-limited chemostat. The v vs s curve for toluene utilization by cells harvested from a *m*-xylene-limited chemostat culture was almost identical at low concentrations of toluene, but maximal conversion rates were 40–50% lower (not shown). Values for the specific affinity (a_A^0 , the slope of the v vs s curve near the origin), the maximal conversion rate ($V_{\max}$) and the half-saturation constant (K_T) were derived from the v vs s curves by fitting the Michaelis–Menten equation to the data points (note: the use of the parameter K_T does not imply the assumption of an active transport system for the hydrocarbons). The v vs

Fig. 1. Influence of the concentration of *m*-xylene (●), *p*-xylene (▲) and toluene (◆) on the rate of conversion (v vs s curve) by cells of *P. putida* (pWW0) harvested from chemostat cultures ($D = 0.05 \text{ h}^{-1}$) as described in the text. The data points were derived from substrate-depletion curves and fitted with the Michaelis-Menten equation by the non-linear least-squares method, yielding data for K_s and $V_{\max}$ as included in Table 1. Inset: expansion of data for lower s values.

s curves appeared to be indistinguishable from linear at low concentrations of the substrate (Fig. 1). Therefore, alternative values for a_A^0 were calculated by linear regression on all data points at substrate concentrations less than 30% of K_T . The values for a_A^0 were found to be approximately 20 times higher for *m*-xylene than for toluene (irrespective of which calculation method was used). *p*-Xylene showed intermediate values for a_A^0 . The $V_{\max}$ values observed for the three hydrocarbons showed no significant differences (based on the single standard errors, see Table 1).

Table 1. Maximal conversion rates ($V_{\max}$), half-saturation constants (K_T) and specific affinities (a_A^0) for *m*-xylene, *p*-xylene and toluene of cells harvested from chemostat cultures of *P. putida* (pWW0)

The values were derived by non-linear least-square fitting of the Michaelis-Menten equation to all data points of the v vs s curves (Fig. 1). Alternative values for a_A^0 were derived by linear regression of the data points with values for v below 30% of $V_{\max}$. Alternative values for $V_{\max}$ were calculated by linear regression on a substrate depletion curve including all data points for which the substrate concentration is more than 300% higher than the K_T value. Values are shown $\pm$ single standard errors. ND, Not determined.

Compound	Calculation method	$V_{\max}$ [mmol h ⁻¹ (g dry wt) ⁻¹]	K_T (μM)	a_A^0 [l (g dry wt) ⁻¹ h ⁻¹]
Toluene	Michaelis-Menten	14.1 $\pm$ 1.0	7.7 $\pm$ 1.7	1820 $\pm$ 240*
	Linear regression	11.2 $\pm$ 0.5	ND	1300 $\pm$ 20
<i>m</i> -Xylene	Michaelis-Menten	12.5 $\pm$ 0.4	0.34 $\pm$ 0.04	36950 $\pm$ 120*
	Linear regression	12.8 $\pm$ 0.2	ND	24700 $\pm$ 960
<i>p</i> -Xylene	Michaelis-Menten	15.7 $\pm$ 0.3	1.3 $\pm$ 0.1	11800 $\pm$ 60*
	Linear regression	14.3 $\pm$ 0.6	ND	9170 $\pm$ 180

* Calculated as $V_{\max}/K_T$ (see Button 1985, 1993).

Fig. 2. Consumption of 3-methylbenzyl alcohol (■) and the transient accumulation of 3-methylbenzoate (●) by a cell suspension (81.7 mg dry wt l⁻¹) of *P. putida* (pWW0) harvested from a *m*-xylene-limited chemostat ($D = 0.05 \text{ h}^{-1}$). The framed numbers indicate the rates of consumption or synthesis expressed as mmol h⁻¹ (g dry wt)⁻¹ as calculated by linear regression on the data points connected by the solid lines. The broken lines show assumed consumption or synthesis patterns.

Flux of intermediates through the TOL pathway

Cell suspensions of *P. putida* (pWW0) were spiked with 35 μM toluene or *m*-xylene, or with 450 $\pm$ 50 μM benzyl alcohol, 3-methylbenzyl alcohol, benzaldehyde or 3-methylbenzaldehyde. The changes in concentrations of these compounds, and all other possible intermediates between the hydrocarbons and the catechols, were monitored. During conversion of toluene, no intermediates were detected. During conversion of *m*-xylene, 3-methylbenzoate was the only intermediate that accumulated at measurable concentrations [at a rate of approximately 2 mmol h⁻¹ (g dry wt)⁻¹]. When the cell

Fig. 3. Consumption of 3-methylbenzaldehyde (▲) and the transient accumulation of 3-methylbenzyl alcohol (■) and 3-methylbenzoate (●) by a cell suspension (104 mg dry wt l⁻¹) of *P. putida* (pVW0) harvested from a *m*-xylene-limited chemostat ($D = 0.05$ h⁻¹). The framed numbers indicate the rates of consumption or synthesis expressed as mmol h⁻¹ (g dry wt)⁻¹ as calculated by linear regression on the data points connected by the solid lines. The broken lines show assumed consumption or synthesis patterns.

Fig. 4. Maximal-flux diagram for *m*-xylene, toluene and their intermediates through the upper pathway and the first step of the *meta*-pathway. The superscript letters indicate the experiments from which the values were derived: a, the experiment depicted in Fig. 1; b, the experiment depicted in Fig. 2; c, the experiment depicted in Fig. 3; d, the maximal consumption rate of 3-methylbenzaldehyde minus the initial formation rate of 3-methylbenzaldehyde measured in the experiment depicted in Fig. 3. The rates for toluene and its intermediates were derived from similar experiments (not shown in figures).

suspension was supplied with 3-methylbenzyl alcohol, 3-methylbenzoate was found to accumulate transiently but no 3-methylbenzaldehyde was detected (Fig. 2).

Fig. 5. Simultaneous consumption of toluene (●) and *m*-xylene (■) by cells harvested from a *m*-xylene-limited chemostat culture.

When the cells were supplied with 3-methylbenzaldehyde, both 3-methylbenzyl alcohol (due to the reverse action of 3-methylbenzyl alcohol dehydrogenase) and 3-methylbenzoate accumulated transiently (Fig. 3). The framed numbers in Figs 2 and 3 refer to the (mean) consumption or synthesis rates of the compounds in the time intervals indicated by the solid lines. Similar patterns were found after pulses of benzyl alcohol and benzaldehyde. The observed rates of consumption and synthesis of substrates and intermediates were used to compose a maximal-flux diagram for the upper pathway and the first step of the *meta*-pathway [the conversion of (methyl)benzoate to the corresponding dihydrodiol] (Fig. 4).

Simultaneous consumption of toluene and *m*-xylene

Cells harvested from the *m*-xylene-limited chemostat culture were supplied with 5 μM toluene and 5 μM *m*-xylene simultaneously and the concentration of these substrates was followed with time. The consumption of *m*-xylene started immediately at a rate comparable to the rate when *m*-xylene was the sole substrate. The consumption rate of toluene was very low until the concentration of *m*-xylene had decreased to below approximately 0.5 μM (Fig. 5). A similar pattern was found with cells harvested from a toluene-limited chemostat (data not shown).

Inhibition of the conversion of *m*-xylene by methylbenzyl alcohol

The $V_{\max}$ for *m*-xylene displayed by cells harvested from a *m*-xylene-limited chemostat was found to be lowered by the presence of methylbenzyl alcohol, the product of the first step of the TOL pathway (Fig. 6). The concentration of methylbenzyl alcohol causing a 50% inhibition of the conversion rate was approximately 300 μM.

Fig. 6. Inhibition of the maximal *m*-xylene conversion rate by methylbenzyl alcohol. The cells used were harvested from a *m*-xylene-limited chemostat ($D = 0.05 \text{ h}^{-1}$).

DISCUSSION

The kinetics of biodegradation of various substrates of the TOL upper pathway were compared. The cells used were harvested from chemostat cultures grown under either *m*-xylene or toluene limitation. These growth conditions were previously found to result in relatively stable and high expression levels of the upper pathway [1250–1500 mU (mg protein)⁻¹ as measured by benzyl alcohol dehydrogenase activity; Duetz *et al.*, 1997).

Individual v vs s curves for toluene, *m*-xylene and *p*-xylene were derived from substrate-depletion curves. The data points at substrate concentrations above K_s fitted well with the Michaelis–Menten equation (Fig. 1). In the area below K_s , however, the relationship between v and s was indistinguishable from linearity, especially for *m*-xylene and *p*-xylene. A similar deviation from Michaelis–Menten kinetics has previously been observed in many cases (reviewed by Dabes *et al.*, 1973; Koch, 1982). Diffusion limitation has been put forward as a possible explanation if the specific affinity (a_A^0 , the slope of the v vs s curve near the origin) observed is relatively high (Koch, 1982). Button (1993) modelled diffusion of substrates to the cell surface. Using his models it can be calculated that cells the size of pseudomonads may attain maximal (diffusion-limited) a_A^0 values for compounds like toluene in the order of 60000 l h⁻¹ (g dry wt)⁻¹. The a_A^0 for *m*-xylene in this study [24700 l h⁻¹ (g dry wt)⁻¹] is close to this theoretical threshold value, indicating that diffusion limitation could play a role. A practical consequence of the linearity of the v vs s curve near the origin is the systematic overestimation of a_A^0 when derived from fitting the data to the Michaelis–Menten equation. We assume therefore that the (22–33% lower) values for a_A^0 derived by linear regression of the data points near the origin (see Table 1) are more reliable than the values derived from fitting all data points to the Michaelis–Menten equation. The a_A^0 observed for *m*-xylene was almost 20-fold higher than the a_A^0 for toluene [1300 l h⁻¹ (g dry wt)⁻¹]. No published data for the a_A^0 for *m*-xylene by pure cultures are available. For toluene, a_A^0 values were determined using cells of *P. putida* F1 (carrying the *tod* pathway) grown in

batch culture (Robertson & Button, 1987). The maximal values for a_A^0 they observed were around 1000 l h⁻¹ (g dry wt)⁻¹. These values for a_A^0 are similar to the data presented in our study but care should be taken in comparing them; apart from the difference in degradation pathway between the two strains, the difference in the cultivation method of the cells should also be taken into account. Earlier studies have shown that cells pre-grown at maximal growth rate exhibit a lower expression level of the catabolic pathways involved (and consequently a lower a_A^0) than cells grown under carbon-limited conditions in chemostat culture (Duetz *et al.*, 1997; Harder & Dijkhuizen, 1983).

The relatively high a_A^0 values for the xylenes (in comparison to that of toluene) found in our study suggest that the role of the TOL pathway in nature, where substrate concentrations are generally low, lies mainly in its capacity to channel xylenes and perhaps other di- or tri-alkylsubstituted benzenes into central metabolism. The sequential utilization of low concentrations of *m*-xylene and toluene when added simultaneously to a cell suspension (Fig. 5) confirms the preferred consumption of the xylenes. A relatively minor role of the TOL pathway in natural toluene degradation was also suggested by our previous finding that *P. putida* (pWW0) was the least competitive strain among four different toluene-degrading soil bacteria when grown under toluene or oxygen limitation (Duetz *et al.*, 1994).

The $V_{\max}$ values for toluene, *m*-xylene and *p*-xylene showed no significant differences, as was also observed by Abril *et al.* (1989). The absolute values for $V_{\max}$ measured were approximately 10 times higher than the flux in the fermenters from which the cells were harvested. Such an overcapacity for the uptake of a growth-limiting substrate is common under carbon- and energy-limiting growth conditions (Harder & Dijkhuizen, 1983). These high specific consumption rates are probably not attained in the natural environment, even when it is heavily contaminated with oil products. This is because the maximal (equilibrium) concentration of toluene and xylenes in the aqueous phase is as low as 1–10 μM due to their tendency to partition in the oil phase (Chapelle, 1993; Kappeler & Wuhrman, 1978). The concentrations encountered by bacterial populations at some distance from the water–oil interface may be substantially lower than the equilibrium concentration due to low diffusion rates.

An additional goal of the present study was to assess to what extent the various enzymic steps control the total flux through the TOL pathway or, in other words, which enzymes may be considered as ‘bottlenecks’. A flux study on the TOL upper pathway is relatively uncomplicated since the intermediates are rather lipophilic and can presumably diffuse passively through the cell membrane. After a pulse of 3-methylbenzyl alcohol, 3-methylbenzoate continued to accumulate in the supernatant until 3-methylbenzyl alcohol was fully utilized (Fig. 2). The subsequent reuptake of 3-methylbenzoate occurred at a rate about 50% of the maximal 3-methylbenzyl alcohol conversion rate. The intermediate,

3-methylbenzaldehyde, did not accumulate at measurable levels (less than $0.5 \mu\text{M}$), indicating that the maximal *in vivo* activity of benzaldehyde dehydrogenase was higher than that of the previous enzyme benzyl alcohol dehydrogenase. This was consistent with the observation that 3-methylbenzaldehyde, after it was pulsed to a cell suspension, was converted to 3-methylbenzoate at a higher rate [$26 \text{ mmol h}^{-1} (\text{g dry wt})^{-1}$; see Fig. 3] than the consumption rate observed for 3-methylbenzyl alcohol [$21.1 \text{ mmol h}^{-1} (\text{g dry wt})^{-1}$, see Fig. 2]. The transient accumulation of 3-methylbenzyl alcohol, as observed after a pulse of 3-methylbenzaldehyde, was probably due to the reverse action of benzyl alcohol dehydrogenase. No accumulation of (methyl)benzoate *cis*-glycol or (methyl)catechol was observed in any experiment (results not shown), suggesting that the *cis*-glycol dehydrogenase and catechol 2,3-dioxygenase steps occurred at rates equal to that of (methyl)benzoate consumption. That this is not always the case was shown by Ampe & Lindley (1995), who observed accumulation of catechol during growth of *Alcaligenes eutrophus* on benzoate.

The 25% faster conversion of benzyl alcohol in comparison to methylbenzyl alcohol is in qualitative agreement with the relative activity of purified benzyl alcohol dehydrogenase on these two substrates (8% faster conversion of benzyl alcohol) as reported by Shaw & Harayama (1990). The same research group found no significant difference between the activities of purified benzaldehyde dehydrogenase on benzaldehyde and 3-methylbenzaldehyde (Inoue *et al.*, 1995), which is also consistent with the present results (Fig. 4). Apparently inconsistent with the present study, however, is their observed K_m value for benzyl alcohol of $220 \mu\text{M}$ (Shaw & Harayama, 1990). If the K_m were similarly high *in vivo*, benzyl alcohols would have accumulated to detectable levels during the conversion of the hydrocarbons, which was actually not the case. A possible explanation is that the K_m value of Shaw & Harayama (1990) is an overestimation, caused by product inhibition, which may not be occurring *in vivo* where benzaldehyde is immediately converted to benzoate.

We may conclude from the flux experiments that the initial monooxygenation step is *in vivo* the slowest step of the upper pathway (see also Fig. 4). This situation may be assumed to be independent of the level of expression since the encoding genes of the upper pathway are organized in a single operon. It may be that the significant overcapacity of the two dehydrogenation steps reflects the limited ability of the strain to actively regulate the ratio between the enzymes. A second possibility is that the relative expression levels of the enzymes of the TOL pathway have actually been optimized in the course of evolution through post-transcriptional regulation systems (e.g. by differences in half-lives of various mRNA regions and enzymes). The function of the relatively high activities of the dehydrogenases could be the fast and efficient removal of (methyl)benzyl alcohol. In this way the degree of product inhibition of xylene monooxygenase is minimized and

so its *in vivo* activity is optimized. A restricted expression of xylene monooxygenase may be essential for the cell in the light of the observation that high expression levels of monooxygenases can cause damage to the cell membrane (Nieboer *et al.*, 1993). This damage, even when not lethal, might lead to an increase in the maintenance-energy requirement and so diminish the competitiveness of the strain.

Whereas the xylene oxygenase may be assumed to limit the flux through the upper pathway independent of the growth conditions, the situation is different with respect to the first enzyme of the *meta* pathway, toluate 1,2-dioxygenase. When, as in the present study, cells are used that were pre-grown under *m*-xylene limitation, the maximal *in vivo* activity of this enzyme was found to be much lower than that of the two previous steps. Under other growth conditions, such as nitrogen, phosphate or sulphate limitation, however, the expression of the *meta* pathway was found to be relatively high in comparison to the upper pathway (Duetz *et al.*, 1997). As a consequence, the activity of toluate 1,2-dioxygenase may not – or may to a lower extent – limit the flux under those growth conditions. On the other hand, the extent to which toluate 1,2-dioxygenase (and also xylene monooxygenase) limits the flux may be stronger under natural conditions (e.g. in soil) since oxygen is generally not present at air-saturated levels as in this study. Suboptimal concentrations of oxygen may have a significant impact on the activity of oxygenases as their K_m values for oxygen are generally rather high ($10\text{--}60 \mu\text{M}$, corresponding to 4–25% of the air-saturated concentration: Shaler & Klecka, 1985).

Kinetic data such as presented here for the TOL pathway could be helpful in modelling the disappearance of the various BTEX compounds as a function of the size and composition of the BTEX-degrading population. The a_A^0 values for various compounds may be of particular assistance in anticipating the residual concentrations that may finally be attained.

ACKNOWLEDGEMENTS

We thank Astrid Mars and Wim de Koning from the University of Groningen for their advice on the application of the substrate-depletion method. This research was supported by an EU-ENVIRONMENT grant (EV5V-CT94-0539).

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Received 8 August 1997; revised 22 November 1997; accepted 17 March 1998.