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

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*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 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 [11-14 mmol h⁻¹ (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 l (g dry wt)⁻¹ 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 Hayashi. 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; Marques 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,
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 ($V_{\text{max}}$, $K_T$, 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 ± 1°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 l⁻¹) or toluene (76 ml l⁻¹), corresponding to an inflow of 24.0 and 25.2 µmol 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 µm 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 H₂SO₄ (86:14, v/v) run at a flow rate of 0.4 ml min⁻¹.

**Biomass concentration.** The OD₅₈₀ 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 OD₅₈₀ values was found; under all conditions tested, an OD₅₈₀ of 1.0 corresponded to a dry weight of 380 ± 10 mg l⁻¹, which was used to estimate the dry weight from the OD₅₈₀ 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 ($V_{\text{max}}$, $K_T$, 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 ($D = 0.05$ 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 µm 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 $\nu$ 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. $\nu$ 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 $\nu$ 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 $\nu$ vs $s$ curve for tolune 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 ($a^\alpha$, the slope of the $\nu$ vs $s$ curve near the origin), the maximal conversion rate ($V_{\text{max}}$) and the half-saturation constant ($K_T$) were derived from the $\nu$ 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 $\nu$ 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 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 $a^0_k$ were calculated by linear regression on all data points at substrate concentrations less than 30% of $K_T$. The values for $a^0_k$ 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 $a^0_k$. 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 ($K_s$) and specific affinities ($a_k^0$) 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 $a^0_k$ 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 $K_s$ value. Values are shown ± single standard errors. ND, Not determined.

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

* Calculated as $V_{max}/K_s$ (see Button 1985, 1993).
Fig. 3. Consumption of 3-methylbenzylaldehyde (△) and the transient accumulation of 3-methylbenzyl alcohol (■) and 3-methylbenzoate (●) by a cell suspension (104 mg dry wt l⁻¹) of P. putida (pWW0) 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).

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.

suspension was supplied with 3-methylbenzyl alcohol, 3-methylbenzoate was found to accumulate transiently but no 3-methylbenzaldehyde was detected (Fig. 2).
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 tolune limitation. These growth conditions were previously found to result in relatively stable and high expression levels of the upper pathway [1250–1500 μM (mg protein)⁻¹ as measured by benzyl alcohol dehydrogenase activity; Duetz et al., 1997].

Individual ν vs s curves for tolune, m-xylene and p-xylene were derived from substrate-depletion curves. The data points at substrate concentrations above Kₘ fitted well with the Michaelis–Menten equation (Fig. 1). In the area below Kₘ, however, the relationship between ν 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 (aₓ, the slope of the ν vs s curve near the origin) observed is relatively high (Koch, 1982). Button (1993) modelled diffusion of substrates to the cell surface. Using his model it can be calculated that cells the size of pseudomonads may attain maximal (diffusion-limited) aₓ values for compounds like tolune in the order of 60 000 l h⁻¹ (g dry wt)⁻¹. The aₓ for m-xylene in this study [125 000 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 ν vs s curve near the origin is the systematic overestimation of aₓ when derived from fitting the data to the Michaelis–Menten equation. We assume therefore that the (22–33 % lower) values for aₓ 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 aₓ observed for m-xylene was almost 20-fold higher than the aₓ for tolune [1300 l h⁻¹ (g dry wt)⁻¹]. No published data for the aₓ for m-xylene by pure cultures are available. For tolune, aₓ values were determined using cells of P. putida F1 (carrying the tod pathway) grown in batch culture (Robertson & Button, 1987). The maximal values for aₓ they observed were around 1000 l h⁻¹ (g dry wt)⁻¹. These values for aₓ 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 aₓ) than cells grown under carbon-limited conditions in chemostat culture (Duetz et al., 1997; Harder & Dijkhuizen, 1983).

The relatively high aₓ values for the xylenes (in comparison to that of tolune) 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-alkyl substituted benzenes into central metabolism. The sequential utilization of low concentrations of m-xylene and tolune 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 tolune degradation was also suggested by our previous finding that P. putida (pWW0) was the least competitive strain among four different tolune-degrading soil bacteria when grown under tolune or oxygen limitation (Duetz et al., 1994).

The Vmax values for tolune, m-xylene and p-xylene showed no significant differences, as was also observed by Abril et al. (1989). The absolute values for Vmax 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 tolune 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 μM), 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 mmol h⁻¹ (g dry wt)⁻¹; see Fig. 3] than the consumption rate observed for 3-methylbenzyl alcohol [21-1 mmol h⁻¹ (g dry wt)⁻¹, see Fig. 2]. The transient accumulation of 3-methylbenzyl alcohol dehydrogenase. This was consistent with the previous results (Fig. 3), which is also consistent with the present results (Fig. 4). Apparently inconsistent with the present study, however, is their observed Km value for benzyl alcohol of 220 μM (Shaw & Harayama, 1990). If the Km 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 Km 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 monooxygenase reaction 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)benzaldehyde. 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 Km values for oxygen are generally rather high (10–60 μM, 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 BTX compounds as a function of the size and composition of the BTX-degrading population. The aₚ values for various compounds may be of particular assistance in anticipating the residual concentrations that may finally be attained.

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