Effect of growth rate, nutrient limitation and succinate on expression of TOL pathway enzymes in response to *m*-xylene in chemostat cultures of *Pseudomonas putida* (pWW0)

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Previous studies have shown that expression of the toluene and *m*- and *p*-xylene degradation pathway in *Pseudomonas putida* (pWW0) is subject to catabolite repression by succinate. We report here that the expression level of the upper part of this so-called TOL pathway in cells grown in chemostat culture is strongly influenced by nutrient limitation when *m*-xylene is the sole carbon and energy source. The benzylalcohol dehydrogenase (BADH) levels in cells that are growth-limited by anabolic processes [sulphate (S)-, phosphate (P)- or nitrogen (N)-limiting conditions] were 3–12% of those in cells growing under oxygen limitation (when catabolism limits growth). BADH levels under S-, P- and N-limitation were further decreased (three- to fivefold) when succinate was supplied in addition to *m*-xylene. Levels of the *meta*-cleavage pathway enzyme catechol 2,3-dioxygenase were less affected by the growth conditions but the general pattern was similar. Dilution rate also influenced the expression of the TOL pathway: BADH levels gradually decreased with increasing dilution rates, from 1250 mU (mg protein)⁻¹ at *D* = 0.05 h⁻¹ under *m*-xylene limitation to 290 mU (mg protein)⁻¹ at *D* = 0.58 h⁻¹ (non-limited growth). BADH levels were shown to be proportional to the specific affinity of whole cells for *m*-xylene. It may, therefore, be expected that natural degradation rates are adversely affected by anabolic nutrient limitations, especially at relatively low concentrations of the xenobiotic compound.

Keywords: catabolite repression, TOL pathway, catechol 2,3-dioxygenase, benzylalcohol dehydrogenase, biodegradation

INTRODUCTION

TOL plasmid pWW0 in *Pseudomonas putida* mt-2 encodes a degradation pathway through which toluene, *m*-xylene, *p*-xylene, pseudocumene and *m*-ethyltoluene are degraded to Krebs cycle intermediates (Assinder & Williams, 1990). The TOL genes are organized in two operons: the upper operon, Pu, encodes the three enzymes responsible for the oxidation of a methyl group of the hydrocarbons to a carboxy group; the *meta* operon Pm encodes the subsequent metabolism of the aromatic carboxylic acids via *meta*-ring cleavage (Assinder & Williams, 1990). Until recently, expression of the TOL genes was considered to be solely dependent

on the efficacy and concentration of the inducer (Abril *et al.*, 1989; Assinder & Williams, 1990).

During the last few years, evidence has accumulated that the presence of growth-saturating concentrations of primary substrates such as succinate or glucose may significantly decrease the inducibility of especially the first enzymes (the upper pathway) of the TOL pathway (Cases *et al.*, 1996; Duetz *et al.*, 1994, 1996; Holtel *et al.*, 1994; Hugouvieux-Cotte-Pattat *et al.*, 1990; Marqués *et al.*, 1994). This catabolite repression effect was shown to be exerted at the transcriptional level (Duetz *et al.*, 1994, 1996; Holtel *et al.*, 1994; Marqués *et al.*, 1994) but the mechanism is still largely unknown.

A recent study on chemostat-grown cells showed that the inducibility of the TOL upper pathway during growth on succinate is highly dependent on the nutrient

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limitation (Duetz *et al.*, 1996). Under strict anabolic growth limitations such as phosphate or sulphate, supply of the non-metabolizable inducer *o*-xylene resulted in only 1–2% of the maximal expression levels that were reached under carbon and energy limitation (Duetz *et al.*, 1996). During growth at maximal rate on succinate, the inducibility of the TOL pathway was reduced to a similar extent to that under anabolic growth limitation (Duetz *et al.*, 1994).

The present study was carried out to determine to what extent these differences in inducibility are related to (a) the presence of a primary substrate such as succinate or (b) other growth parameters such as the nutrient limitation and the growth rate. Therefore, we measured the expression levels of the TOL pathway under different limitations with either *m*-xylene as sole carbon and energy source (C-source) or with both *m*-xylene and succinate as 'mixed' substrates. In addition, the expression of the TOL pathway was measured at various dilution rates with *m*-xylene as sole C-source. *m*-Xylene was used as a substrate as it was found to support a higher maximal growth rate than did toluene; we therefore consider *m*-xylene to be a more 'natural' substrate than toluene for the TOL pathway. The specific activities of the second enzyme of the upper pathway, benzylalcohol dehydrogenase (BADH), and the third enzyme of the *meta*-pathway, catechol 2,3-dioxygenase (C23O) were used as measures for the expression levels of Pu and Pm, respectively. It was found that the specific condition of nutrient limitation had a stronger effect on the expression level of both enzymes than did the presence or absence of the primary substrate succinate.

METHODS

Bacterial strain. *P. putida* mt-2 (ATCC 33015) harbouring the TOL plasmid pWW0 was a gift of P. A. Williams (Bangor, UK).

Media. The composition of the mineral-salts medium (MSM) was varied depending on the nutrient limitation required (for concentrations of the inorganic nutrients, see Duetz *et al.*, 1996). The limiting conditions applied were oxygen-limitation, carbon and energy limitation (C-limitation), ammonium-limitation (N-limitation), phosphate-limitation (P-limitation) or sulphate-limitation (S-limitation). When applicable, MSM was supplemented with either 10 mM succinate for succinate-limited growth, or 20 mM succinate for other nutrient limitations.

Chemostat culture. The fermenters used were custom-made, shaped like an Erlenmeyer flask, and had a working volume of 100–110 ml. Further details on the fermenter, stirring speed and aeration rate under O_2 -limitation and other limitations have been described previously (Duetz *et al.*, 1996). The temperature was maintained at $28 \pm 1^\circ\text{C}$. The standard procedure for starting a chemostat culture was to grow the cells initially under succinate-limitation at $D = 0.05\text{ h}^{-1}$ until a steady state was reached (3–5 d), and to shift to *m*-xylene-limiting conditions subsequently. When a new steady state was established, the chosen nutrient-limitation was imposed on the culture by supplying the appropriate MSM.

Supply of volatile C-sources. When applicable, *m*-xylene was supplied to the chemostat culture by supplementing the incoming airflow (6 l h^{-1}) with air saturated (at 0°C) with *m*-xylene at a rate dependent on the imposed nutrient limitation and the dilution rate: 300 ml h^{-1} to achieve *m*-xylene-limiting conditions at $D = 0.05\text{ h}^{-1}$ and three- to fivefold higher for other limitations. For growth at dilution rates greater than 0.05 h^{-1} , the supply of *m*-xylene-saturated air was increased in proportion with the dilution rate. The exact concentration of *m*-xylene in the inlet air was calculated from the rates of *m*-xylene evaporation into the gas-flow and the total airflow. The concentration of *m*-xylene in the headspace of the fermenter was derived from this value by multiplication with the quotient of the GC peak areas (see below) of gas sampled from the headspace and the inlet air, respectively. To prevent losses of *m*-xylene by adsorption or diffusion, all material used in the gas supply system was made either of stainless steel or butyl rubber.

Continuous cultivation at maximal growth rate. To achieve maximal growth rate conditions, the dilution rate of a chemostat culture, growing initially under *m*-xylene-limitation at $D = 0.5\text{ h}^{-1}$, was increased to 0.58 or 0.69 h^{-1} (in two separate experiments). At the same time, the supply rate of *m*-xylene was doubled to establish growth-saturating concentrations of *m*-xylene. Subsequently, the change in biomass concentration (X) with time was followed and a value for the non-limited growth rate ($\mu_{\max}$) was calculated using the equation (Button, 1993):

$$\mu_{\max} = D + \frac{1}{t} \ln \frac{X}{X_0} \quad (1)$$

Analysis of *m*-xylene in the gas phase. The percentage of *m*-xylene converted in the fermenter was calculated from the peak areas resulting from the injection of $250\text{ }\mu\text{l}$ samples of the incoming air and the headspace of the fermenter into a Hewlett Packard type 5890 gas chromatograph equipped with an HP101 methylsilicone capillary column and a split injector (1:7) (both at 100°C) and a flame ionization detector. A gas-tight glass syringe (SGE) with a stainless steel plunger was used for injection.

Analysis of *m*-xylene and succinate in the liquid phase. Samples were taken from a fermenter and filtered through a $0.22\text{ }\mu\text{m}$ filter within 5 s. For *m*-xylene analysis, samples of $10\text{ }\mu\text{l}$ were injected into an HPLC apparatus equipped with a variable-wavelength detector (Hewlett Packard 1050 series) set at 195 nm . The solvent was acetonitrile/water 86/14 [% v/v] run at a flow rate of 0.4 ml min^{-1} in a 10 cm Hypersil C18 column (Chrompack). For succinate analysis, samples of $1\text{ }\mu\text{l}$ were analysed with the same equipment using a 20 cm Chromspher C18 column (Chrompack) and $5\text{ mM H}_2\text{SO}_4$ in water as a solvent. Detection of succinate was at 207 nm .

Enzyme assays. Extracts of culture cells sampled from the fermenters were prepared by sonication in 100 mM phosphate buffer, pH 7.5 containing 10% acetone at 4°C and subsequent centrifugation at 40000 g . BADH and C23O activities were determined as described by Worsey & Williams (1975) and Sala-Trepat & Evans (1971), respectively.

Protein determination. Protein content in cell extracts was determined according to a modified Lowry method (Petersen, 1977).

Oxygen consumption rates by whole cells. Cells were harvested from a steady-state culture, washed with a physiological salts solution, and resuspended in MSM without any

C-source. The cell suspension was subsequently tested for oxygen uptake before and after the addition of 10 μ M *m*-xylene, using a Clark oxygen electrode (type YSO LN2555, Yellow Springs Instruments) and a high-sensitivity membrane from the same supplier.

Biomass concentration. The optical density at 540 nm (OD_{540}) of chemostat cultures was measured regularly with a Beckman 40 spectrophotometer. In a previous study by Duetz *et al.* (1996), no important influence of the growth condition was found for the relationship between the data for the dry weight and OD_{540} values. Under all conditions tested, an OD_{540} of 1.0 correlated with a mean dry weight of 380 mg l⁻¹. This value was used to estimate the dry weight from the OD_{540} data.

Assay for TOL function loss. At regular intervals (2–3 d), samples were withdrawn from the chemostat, diluted and plated onto peptone agar. After 2 d of growth, the colonies were checked for the presence of the TOL pathway by the catechol-spraying method (Duetz *et al.*, 1991).

Determination of the uptake kinetics of *m*-xylene. A 21 ml glass headspace vial (Hewlett Packard) was filled with 1.5–1.95 ml of MSM and supplied with a steel stirring bar (18 $\times$ 5 mm). The vial was closed by a Teflon-lined rubber septum and supplied with 250–500 μ l *m*-xylene-saturated air (at 0 °C) to result in a *m*-xylene concentration in the headspace of 1–2 μ M. Subsequently, the vial was kept at 28 °C and stirred magnetically at 1300 r.p.m. A cell suspension (50–500 μ l), freshly harvested from a chemostat culture, was added using a syringe. The resulting decrease of the concentration of *m*-xylene in the headspace was monitored by injecting 250 μ l into a gas chromatograph (as described above) at regular time intervals (each 2–4 min). The concentration of *m*-xylene in the liquid phase (*s*) was calculated from the headspace data assuming an equilibrium between the two phases and a dimensionless Henry coefficient of 0.3 (Eastcott *et al.*, 1988). For each time interval, the specific *m*-xylene consumption rate (*v*) was calculated assuming a linear decrease of the *m*-xylene concentration. The resulting values for *v* were plotted against the mean of the *m*-xylene concentrations at both ends of the time interval. The systematic underestimation of *v*, inherent in this mathematical procedure, was calculated to be less than 3% for all data points presented in this study and was therefore neglected. The values for *v* were corrected for the amount of *m*-xylene present in the gas phase (74% of the total amount of *m*-xylene present in the vial). The maximal consumption rate (V_{max}) was taken as the mean of the values for *v* determined at substrate concentrations more than three times the concentration at $\frac{1}{2}V_{max}$. The specific affinity (a_A^0), representing the slope of the plot of *v* versus *s* near the origin, was derived by linear regression of all data points for which *v* was between 5 and 50% of V_{max} using the equation (Button, 1985):

$$v = a_A^0 \times s \quad (2)$$

A value for the half-saturation constant (K_T) was calculated using the equation:

$$K_T = \frac{V_{max}}{2a_A^0} \quad (3)$$

The procedure described above assumes a linear relationship between *s* and *v* when *v* is lower than 50% of V_{max} . In addition, the data points were fitted with the Michaelis–Menten equation (Robinson & Characklis, 1984), yielding alternative values for V_{max} and the half-saturation transport constant K_T . The latter parameters also allow an alternative

value for a_A^0 ($a_A^{0'}$) to be calculated, using the equation (Button, 1993):

$$a_A^{0'} = \frac{V_{max}}{K_T} \quad (4)$$

RESULTS

Effect of the growth rate on BADH and C23O levels with *m*-xylene as sole C-source

P. putida (pWW0) was grown in chemostat culture under C-limitation with *m*-xylene as sole C-source. The initial dilution rate was 0.05 h⁻¹. When a steady state was established (when the OD_{540} was constant for at least three generation times) BADH and C23O levels in cell-free extracts were determined and the dilution rate was increased to 0.2 h⁻¹. A new steady state was established in 2 d, and BADH and C23O levels were determined again. After transitions to 0.3 and 0.5 h⁻¹, respectively, steady-state conditions were reached within 1 d. Subsequently, the dilution rate was increased to 0.69 h⁻¹ and the rate of supply of *m*-xylene was doubled, resulting in non-limiting growth conditions. From the resulting decrease of the biomass concentration with time, and using equation (1) from Methods, the maximal growth rate on *m*-xylene was estimated to be 0.58 h⁻¹. When, in a separate duplicate chemostat culture, the dilution rate was set at this value (0.58 h⁻¹), the optical density remained constant (less than 10% variation) for three generation times. It should be noted that this culture – in spite of its constant optical density – cannot be considered to be in a ‘true’ steady state (which is intrinsically impossible). However, the latter data seem to confirm the reliability of the maximal growth rate on *m*-xylene derived from the washout-curve as described above. Both BADH and C23O levels were found to decrease with increasing dilution rate (see Fig. 1). The

Fig. 1. Effect of growth rate on the expression levels of BADH (▲) and C23O (■) in cells of *P. putida* (pWW0) grown in a chemostat culture supplied with *m*-xylene as sole C-source and a mineral medium composed for C-limiting conditions. Activities are expressed as mU (mg protein)⁻¹.

Table 1. Growth characteristics of steady-state chemostat cultures of *P. putida* (pWW0) run at different dilution rates with *m*-xylene as sole carbon and energy source

At dilution rates below the maximal growth rate (0.58 h^{-1}), the concentration of *m*-xylene was growth-limiting.

$D \text{ (h}^{-1}\text{)}$	Residual [<i>m</i> -xylene] (μM)	<i>m</i> -Xylene consumption [$\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$]	Dry wt (mg l^{-1})	$Y \text{ [g dry wt (g } m\text{-xylene)}^{-1}]$
0.05	0.04*	1.3	201	0.37
0.2	< 0.2†	4.6	224	0.41
0.3	< 0.2†	5.8	228	0.49
0.5	< 0.2†	7.3	175	0.64
0.58‡	24	10.2	114	0.64

* Below detection limit of HPLC, value estimated from specific affinity and flux in chemostat.

† Below detection limit of HPLC.

‡ Maximal growth rate on *m*-xylene.

growth yield was found to increase at higher dilution rates (Table 1).

Effect of nutrient limitation on BADH and C23O levels with *m*-xylene as sole C-source

To determine the effect of nutrient limitation on expression of the TOL pathway with *m*-xylene as sole C-source, *m*-xylene-limited chemostat cultures ($D = 0.05 \text{ h}^{-1}$) were supplied with mineral media containing growth-limiting concentrations of sulphate, phosphate or ammonium. When excess limiting nutrient was washed out from the fermenter (all inorganic nutrients are present in more than 10-fold excess in the initial medium) the amount of *m*-xylene was increased to result in (non-growth-limiting) concentrations of *m*-xylene of 6–11 μM (see Table 2). Under N-, P- or S-limitation this

Fig. 2. Effect of nutrient limitation on the expression of the TOL upper pathway in cells grown in chemostat culture ($D = 0.05 \text{ h}^{-1}$) with either solely *m*-xylene (□) or both *m*-xylene and succinate (■) as C-sources, as measured by BADH activity. Activities are expressed as $\text{mU (mg protein)}^{-1}$.

shift resulted in a decrease in the specific activities of BADH. Within 3 d, the enzyme activities became constant (no significant differences between the activities measured on day 2 and day 3 after the increase in the *m*-xylene supply). Steady-state values for the BADH-levels were 8–32 % of those under *m*-xylene-limiting conditions (Fig. 3). Transition of a *m*-xylene-limited culture to O₂-limitation caused the opposite effect: BADH levels increased by more than 160 % (Fig. 2). C23O levels were affected by nutrient limitation to a lesser extent, except for C23O levels under O₂-limitation which were also more than 100 % higher than under other limitations tested (see Fig. 3).

Effect of presence of succinate on BADH and C23O levels and *m*-xylene consumption rates

The effect of the presence of growth-saturating concentrations of succinate in addition to *m*-xylene was quantified for N-, P-, S-, C- and O₂-limiting conditions.

Table 2. Growth characteristics of steady-state chemostat cultures ($D = 0.05 \text{ h}^{-1}$) of *P. putida* (pWW0) growing under different limitations with *m*-xylene as sole carbon and energy source

Limitation	Residual [<i>m</i> -Xylene] (μM)	<i>m</i> -Xylene consumption [$\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$]	Dry wt (mg l^{-1})	$Y \text{ [g dry wt (g } m\text{-xylene)}^{-1}]$
O ₂	26	1.6	125	0.29
C	0.04*	1.3	201	0.37
N	8.8	2.9	167	0.16
P	6.8	2.9	125	0.16
S	11	3.7	190	0.13

* Below detection limit of HPLC: value estimated from specific affinity and flux in chemostat.

Fig. 3. Effect of nutrient limitation on the expression of the TOL meta pathway in cells grown in chemostat culture ($D = 0.05 \text{ h}^{-1}$) with either solely *m*-xylene (□) or both *m*-xylene and succinate (■) as C-sources, as measured by C23O activity. Activities are expressed as mU (mg protein)⁻¹.

Fig. 4. Effect of nutrient limitation on the *m*-xylene oxidation rate of cells harvested from chemostat cultures ($D = 0.05 \text{ h}^{-1}$) with either solely *m*-xylene (□) or both *m*-xylene and succinate (■) as C-sources, as measured by the increase in the oxygen consumption of washed cells after the addition of $10 \mu\text{M}$ *m*-xylene. Oxygen uptake rates are expressed as mmol O₂ (g dry wt)⁻¹ h⁻¹.

Table 3. Growth characteristics of steady-state chemostat cultures ($D = 0.05 \text{ h}^{-1}$) of *P. putida* (pWW0) growing under different limitations with succinate and *m*-xylene as carbon and energy sources

Limitation	Residual [<i>m</i> -xylene] (μM)	Residual [succinate] (mM)	Dry wt (mg l ⁻¹)
O ₂	29	16.7	68
C	< 0.2*	< 0.1*	296
N	11.6	12.6	163
P	22.5	7.0	110
S	15.1	11.1	106

* Below detection limit of HPLC.

Steady-state conditions were reached starting from cultures pregrown under the appropriate nutrient limitation with succinate as sole C-source (Table 3).

Steady-state levels of BADH were decreased most by the presence of succinate under P-limitation (20% of the levels with *m*-xylene as sole C-source), while levels under O₂-limitation decreased to only 54% (Fig. 2). The presence of succinate caused a minor decrease of C23O levels under N-, P- and S-limitation and a small increase under O₂-limitation (Fig. 3). Under C-limitation, the percentage of *m*-xylene in the total supply of C-source was set at 42% (C-mol/C-mol). Both C-sources were found to be degraded to levels below the detection limit (Table 3). BADH and C23O levels decreased to 37 and 32% of the levels measured in cells grown under the same conditions with *m*-xylene as sole C-source (Figs 2 and 3).

Oxygen consumption rates of whole cells upon an *m*-xylene pulse

To establish the relevance of BADH levels as a measure of the expression of the *m*-xylene-degrading capacity of the cells, we harvested and washed cells from the chemostat cultures described above and measured their initial oxygen consumption rates after the addition of $10 \mu\text{M}$ *m*-xylene. These oxygen consumption rates and BADH levels in cell extracts showed similar trends with respect to the influence of the growth conditions (compare Figs 2 and 4).

Maximal conversion rates and specific affinities for *m*-xylene of chemostat-grown cells

The relationship between *m*-xylene consumption rate and *m*-xylene concentration was derived from substrate-depletion curves made with cells grown under S-limitation or C-limitation with *m*-xylene as sole C-source. As expected, the maximal conversion rate of *m*-xylene in cells grown under S-limitation [$2.9 \text{ mmol h}^{-1} (\text{g dry wt})^{-1}$] was similar to the steady-state *m*-xylene conversion rate in the chemostat culture [$3.6 \text{ mmol h}^{-1} (\text{g dry wt})^{-1}$]. The cells grown under C-limitation however showed a maximal conversion rate of *m*-xylene of $9.9 \text{ mmol h}^{-1} (\text{g dry wt})^{-1}$, which is 660% more than the actual flux in the fermenter (Tables 2 and 4). The relationship between V and the concentration of *m*-xylene did not follow Michaelis–Menten kinetics below 50% of V_{max} . Instead, the shape of the curve in this area appeared to be linear. As a result, the data for the specific affinity for *m*-xylene (a_A^0) derived from fitting with the Michaelis–Menten equation (data not shown) were approximately 50% higher than the data derived from linear fitting. We consider the linear-regression data to be more relevant to estimate the conversion rates at low substrate concentrations and therefore ignored

Table 4. Maximal conversion rates ($V_{\max}$), half saturation constants (K_T) and specific affinities (a_A^0) for *m*-xylene of cells harvested from chemostat cultures ($D=0.05\text{ h}^{-1}$) under conditions of C- or S-limitation with *m*-xylene as sole C-source

Values for a_A^0 and K_T were derived by linear regression of the datapoints with values for V below 50% of $V_{\max}$.

Limitation	$V_{\max}$ [mmol h ⁻¹ (g dry wt) ⁻¹]	K_T (μM)	a_A^0 [l (g dry wt) ⁻¹ h ⁻¹]
C	9.9 ± 0.9	0.20	24700 ± 960
S	2.9 ± 0.1	0.59	2450 ± 110

the values derived from fitting with the Michaelis-Menten equation. The specific affinity for *m*-xylene (a_A^0) was higher for cells grown under C-limitation than for cells grown under S-limitation (Table 4). On the basis of a_A^0 and the steady-state conversion rate of *m*-xylene in the chemostat (Table 2), we estimate the residual concentration of *m*-xylene in the C-limited fermenter to be 0.04 μM.

DISCUSSION

In this study, we investigated the influence of the growth conditions on the expression of the TOL pathway in *P. putida* (pWW0). We measured the activity of the upper-pathway enzyme BADH and the *meta*-pathway enzyme C23O in cell-free extracts, as well as the oxygen consumption by washed cells upon the addition of *m*-xylene. We varied three parameters, namely growth rate, nutrient limitation and the C-source.

Effect of growth rate

Growth rate was varied by adjusting the dilution rate of C-limited chemostat cultures fed with MSM and supplied with *m*-xylene as sole C-source. The growth yield appeared to increase significantly at higher growth rates; the yield at a growth rate of 0.4 h⁻¹ was 70% higher than at a growth rate of 0.05 h⁻¹. This is a common phenomenon (also during growth on succinate; Duetz *et al.*, 1994) and may be related to the relatively large proportion of C-source required for maintenance at a low growth rate (Stouthamer & Bettenhausen, 1973). The BADH activity decreased with increasing dilution rates (Fig. 1). Many catabolic enzymes show a similar increase in specific activity when lowering the dilution rate under C-limitation (Harder & Dijkhuizen, 1983; Matin, 1981; Matin *et al.*, 1976). The mechanism underlying this phenomenon is unclear. According to general chemostat theory (Matin, 1981), the residual concentration of the limiting nutrient (*m*-xylene) increases when the growth rate increases, theoretically resulting in a higher degree of saturation of the regulatory XylR protein and thus a higher transcription rate from the upper pathway promoter Pu. However, this is not the case. Apparently, other (unknown)

mechanisms – counteracting the presumed higher degree of saturation of the XylR protein – are active.

Effect of nutrient limitation

Nutrient limitation appeared to exert a strong effect on the expression level of the upper pathway, even when *m*-xylene was the sole C-source (Fig. 2). Under (partly) catabolic limitations such as C- and O₂-limitation, BADH levels were higher than under strict anabolic limitations (N-, P-, and S-limitation). Similarly to the influence of growth rate on TOL expression levels, this pattern is opposite to the effect expected on the basis of the inducer concentration: the actual concentration of *m*-xylene is more than 100-fold higher under S-, P- and N-limitation (7–11 μM) than under C-limited conditions (approx. 0.04 μM). Seemingly, *m*-xylene – when present in excess – may act as a repressor of its own catabolic pathway, via its metabolites or through the higher energy status resulting from their oxidation. These findings support Magasanik (1961) who postulated that ‘any compound which can serve efficiently as a source of intermediary metabolites, and of energy, may reduce the rate of formation of catabolite-repression sensitive enzymes’. The observation that the presence of a growth saturating concentration of *m*-xylene does not cause repression under O₂-limitation (Fig. 2) may be related to relatively low substrate turnover of xylene monooxygenase and C23O due to low oxygen concentration. BADH levels in cells grown under O₂-limitation [3300 mU (mg protein)⁻¹] were approximately the same as those in succinate-limited cells exposed to 3 or 20 μM *o*-xylene (Duetz *et al.*, 1996). We consider both conditions to be optimal for maximal induction: the inducer is present at a concentration that saturates the XylR protein (Duetz *et al.*, 1996), and cells are limited in their growth by their catabolism, causing a general derepression of all catabolic systems (Harder & Dijkhuizen, 1983). The 62% lower activity under *m*-xylene-limitation [1250 mU (mg protein)⁻¹] may be caused by the insufficiency of the residual concentration of *m*-xylene (approx. 0.04 μM) to saturate the XylR regulatory protein.

As discussed above, the mechanism causing the high expression levels of the TOL pathway under conditions

of catabolic nutrient limitation (O_2 - and C-limitation) remains unclear. It is, however, a common phenomenon and high levels of catabolic enzymes have been shown previously to result in a selective advantage to organisms at low C-source concentrations (Harder *et al.*, 1977). In the present study, the ecophysiological function is clearly demonstrated by the differences in the kinetics of *m*-xylene uptake by cells pregrown under C- or S-limitation. The cells pregrown under C-limitation have a specific affinity 10-fold higher than that of cells grown under S-limitation (Table 4). As indicated by Button (1985), the specific affinity is the kinetic parameter that best describes the ability of micro-organisms to accumulate substrate and grow at concentrations below the affinity constant. According to Button (1985), the specific affinity for a lipophilic substrate for which no specific transport system exists is proportional to the amount of rate-limiting enzyme in the cell membrane. Xylene monooxygenase is situated in the cell membrane (Suzuki *et al.*, 1993) and so its level in the membrane is probably an important factor in this respect. We have not measured the levels of xylene monooxygenase directly but, as its gene is part of the same operon as that encoding BADH, it seems reasonable to assume that the xylene monooxygenase level increases in parallel with BADH levels, thus contributing to the high specific affinity for *m*-xylene of C-limited cells. The high expression levels of the TOL pathway under O_2 -limitation probably have a similar ecological function. The consumption rate of *m*-xylene under O_2 -limitation is probably confined by the *in vivo* activity of the oxygenases of the TOL pathway since the Michaelis constants for oxygen of oxygenases (9–62 $\mu M O_2$; Shaler & Klecka, 1985) are generally higher than those for the electron transport chain (0.3–1.3 $\mu M O_2$; Longmuir, 1954). Therefore, we assume that the specific affinity of the cells for oxygen during conversion of *m*-xylene also increases with higher levels of xylene monooxygenase. The overexpression of the TOL pathway under C- and O_2 -limitation causes a V_{max} for *m*-xylene much higher than the flux in the chemostat cultures. The lower V_{max} value for S-limited cells (Table 4), resulting from lower expression levels of the TOL pathway, is probably related to the need for cells growing under anabolic limitation to minimize the amount of protein spent on catabolic enzymes, but possibly also to the need to prevent accumulation of toxic intermediates such as benzaldehydes and catechols.

Catabolite repression by succinate

Under N-, P- and S-limitation, the additional presence of growth-saturating concentrations of succinate resulted in a three to fivefold decrease in the BADH levels when compared to BADH levels under equivalent conditions with *m*-xylene as sole C-source (see Fig. 2). BADH levels were similar to those of cells grown under similar conditions in the presence of the non-metabolizable inducer *o*-xylene (Duetz *et al.*, 1996). Apparently, *m*-xylene and *o*-xylene have a similar inducing strength. Expression of the upper and *meta* pathway under

oxygen-limitation in response to *m*-xylene was found to be only slightly affected by the presence of succinate. Despite the relatively high expression levels of the TOL pathway, the conversion rate of *m*-xylene in the fermenter in the presence of succinate was very low under O_2 -limitation. We assume this to be related to the affinity for oxygen of the first enzyme of the TOL pathway, xylene monooxygenase, which may be too low for a significant transformation rate of *m*-xylene at the prevailing oxygen concentration. As opposed to most other results in this study, there seems to be no apparent ecological function for the high expression levels of the TOL pathway under O_2 -limitation in the presence of growth-saturating concentrations of succinate.

Practical implications

If we assume that the phenomena found for *P. putida* (pWWO) also occur in members of natural populations of xenobiotic-degrading strains, our data may be of assistance in predicting the natural degradation rates as a function of population composition, nutrient limitation and primary C-sources. Strict anabolic nutrient limitations (e.g. P-limitation, which often occurs in nature) may thus be expected to be unfavourable in two ways: (a) the residual concentration of the xenobiotic is expected to remain relatively high (due to a low specific affinity for the xenobiotic); (b) the susceptibility to catabolite repression by primary C-sources is high, which may cause an additional decrease in the conversion rates of the xenobiotics involved.

The observed dependence of the expression level of the TOL pathway enzymes on the growth rate and the nutrient limitation has a number of implications for physiological or kinetic studies done on catabolic systems. Firstly, the determination of the specific affinity for a growth substrate using exponential-phase cells from batch culture may underestimate the potential specific affinity because the catabolic system is partially repressed during non-limited growth (Fig. 1). Furthermore, when growth is slowing down at the end of exponential phase, this lower growth rate by itself may lead to a derepression of the catabolic system studied. Therefore, during growth on mixed substrates an observed increase in the expression level of a catabolic pathway at the end of the exponential phase is no definitive proof for the susceptibility of the pathway to catabolite repression. On the other hand, the extent of catabolite repression may be underestimated when cells are O_2 -limited (often occurring in batch culture), a condition during which almost no catabolite repression was found.

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