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Inducibility of the TOL Catabolic Pathway in *Pseudomonas putida*(pWW0) Growing on Succinate in Continuous Culture: Evidence of Carbon Catabolite Repression Control

WOUTER A. DUETZ,[1]* SILVIA MARQUÉS,[2] CAROLINE DE JONG,[1] JUAN L. RAMOS,[2] AND JOHAN G. VAN ANDEL[1]

Laboratory for Waste Materials and Emissions, National Institute of Public Health and Environmental Protection, 3720 BA Bilthoven, The Netherlands,[1] and Estación Experimental del Zaidin, Department of Plant Biochemistry, Consejo Superior de Investigaciones Científicas, 18080 Granada, Spain[2]

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The TOL catabolic genes in *Pseudomonas putida*(pWW0) are clustered in the upper operon, encoding enzymes for the conversion of toluene and xylenes to benzoate and toluates, and the *meta*-cleavage operon, encoding enzymes for the conversion of the benzoate and toluates to tricarboxylic acid cycle intermediates. In this study, it was shown that cells growing in a chemostat under succinate growth-limiting conditions express both the upper and *meta*-cleavage pathways in response to *o*-xylene, a nonmetabolizable effector of the XylR regulatory protein. The dilution rate maintained in the succinate-limited chemostat cultures influenced the synthesis levels of TOL pathway enzymes, their steady-state levels, and their turnover rates. Cells growing in the presence of nonlimiting concentrations of succinate in continuous culture did not express pathway enzymes in response to the addition of *o*-xylene, which was due to a blockage at the transcriptional level. Expression of the *meta*-cleavage pathway in response to 2,3-dimethylbenzoate, a nonmetabolizable effector of the XylS regulatory protein, was 93% lower in cultures exposed to succinate at nonlimiting concentrations than in the succinate-limited chemostats. The mRNA level of *xylS* during nonlimited growth on succinate was very low compared with that in succinate-limited cultures, suggesting that suppression of expression of the *meta*-cleavage pathway is regulated mainly by the level of the XylS regulator.

Many soil bacteria are known to be capable of mineralizing aromatic compounds. The most extensively studied organism in this respect is *Pseudomonas putida*(pWW0), which can utilize toluene, *m*- and *p*-xylene, pseudocumene, and *m*-ethyltoluene as sole sources of carbon and energy. The genetic information for the transformation of these aromatics to central metabolites is harbored by the transmissible TOL plasmid pWW0 (3). Figure 1 shows the genetic organization of the catabolic operons. Expression of the structural genes on TOL plasmid pWW0 is controlled by two positively acting regulatory proteins, XylR and XylS (3). The XylR protein, which is produced constitutively, interacts with the hydrocarbon substrates and their alcohol metabolites and may in this way become effective as an activator of the upper part of the TOL pathway by promoting transcription from the Pu promoter (2). The ability of the second regulatory protein, XylS, to activate the Pm promoter, which controls expression of the lower part of the TOL pathway, is strongly enhanced by the binding of (methyl-substituted) benzoic acids. The activated XylR protein also promotes transcription from the Ps promoter to render high levels of XylS protein, which, in turn, lead to high-level expression from the Pm promoter independently of XylS protein effectors (3, 15, 18). Both the XylR and XylS proteins exhibit a rather broad effector specificity, being able to recognize as effectors not only pathway substrates but also similar, nonmetabolizable compounds like *o*-xylene for the XylR protein (2) and 2,3-dimethylbenzoate (2,3-DMB) for the XylS protein (19).

Although the regulation of expression has been well studied, little is known about the extent to which induction of the TOL pathway is repressed by the presence of other carbon sources, i.e., is subject to catabolite repression. The first indication of TOL pathway susceptibility to catabolite repression came from the observation of Worsey and Williams in 1975 that cells grown in batch culture on a mixture of acetate and *m*-xylene showed twofold-lower levels of the upper pathway enzymes, benzylalcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH), than those of cells grown with *m*-xylene as the sole source of carbon and energy (23). Later it was demonstrated that the presence of glucose in a batch culture containing *m*-methylbenzyl alcohol resulted in a delay of full induction of the TOL pathway until the late exponential phase (10), indicating a diauxic growth pattern.

The goal of this study was to assess the effect of succinate, a primary carbon and energy source for pseudomonads (8), on the inducibility of the TOL catabolic genes in *P. putida*(pWW0) grown in continuous culture. It was shown that the TOL genes in cells growing on limiting concentrations of succinate were expressed to high levels in response to *o*-xylene. When succinate was supplied at a nonlimiting concentration however, *o*-xylene did not cause any expression of the TOL genes.

(A preliminary report of this work has been presented previously [4].)

* Corresponding author. Mailing address: Laboratory for Waste Materials and Emissions, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Phone: (31)30743063. Fax: (31)30293651. Electronic mail address: LAEWD@LAE01.RIVM.NL.

MATERIALS AND METHODS

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

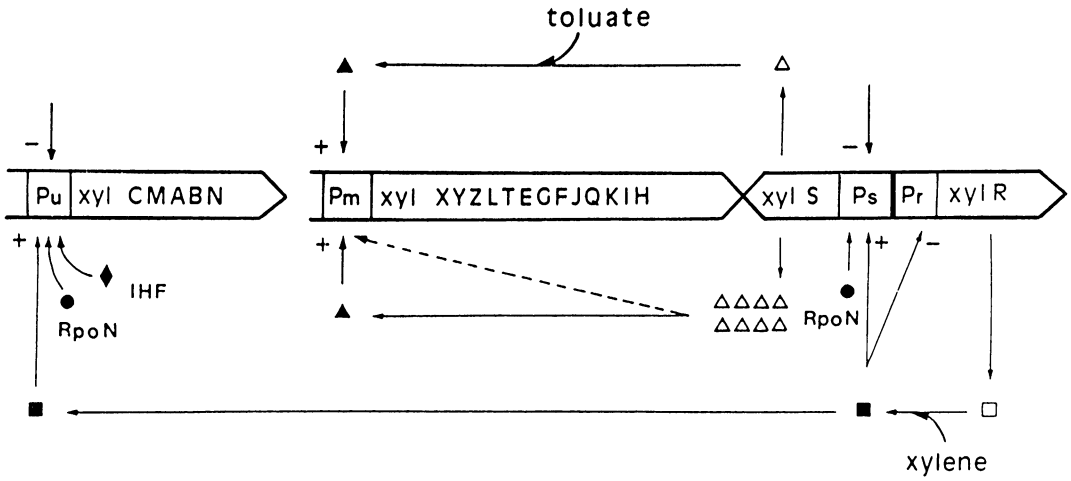

FIG. 1. Genetic organization of the TOL plasmid pWW0 upper and *meta*-cleavage pathways. The regulatory loops are explained in the introduction. +, stimulation of transcription; −, inhibition of transcription (modified from references 15 and 18). IHF, integration host factor.

Media. The growth medium was a mineral salts medium described previously (5), supplemented with (i) 10 mM succinate when supplied to a carbon- and energy-limited culture or (ii) 15 mM succinate when supplied to a (nonlimited) phauxostat culture. Both media contained a 50 mM phosphate buffer set at pH 6.4. When indicated, the medium was supplied with 1 mM 2,3-DMB or 5 mM *m*-methylbenzylalcohol.

Continuous culture. For chemostat culture at the lowest dilution rate (D = 0.05 h^{-1}), fermentors (500 series; L & H Engineering) with a working volume of 500 ml were used. The culture was stirred at 700 rpm. Air was supplied at a rate of 500 ml min^{-1}. Custom-made fermentors, shaped like an Erlenmeyer flask with a working volume of 100 ml, were used for chemostat culture at a dilution rate of 0.5 h^{-1} and for phauxostat culture (5, 6). These cultures were stirred magnetically at 900 rpm, and air was supplied at 100 ml min^{-1}. In both types of fermentors, the temperature was maintained at 28 ± 1°C. Conversion of all succinate under growth-limiting conditions led to an increase of pH of the growth medium to 7.0 ± 0.1. In phauxostat cultures, the pH was maintained at 7.0, resulting in a residual succinate concentration of 4 mM, as described previously (5). When applicable, *o*-xylene or toluene was supplied to the cultures through the gas phase by using an additional airflow, containing 3 μmol of *o*-xylene $liter^{-1}$ or 35 μmol of toluene $liter^{-1}$ through the culture. The flow rates were 33 ml h^{-1} for 100-ml cultures and 170 ml h^{-1} for 500-ml cultures.

Analysis of *o*-xylene and toluene. Samples taken from a fermentor were filtered through a 0.22-μm-pore-size bacterial filter. Samples of 10 μl were injected in a high-performance liquid chromatography apparatus equipped with a fluorescence detector (Shimadzu RF535); wavelengths of excitation and emission were 260 and 290 nm for *o*-xylene and 250 and 280 nm for toluene. The solvent was acetonitrile-water (85/15 [vol/vol]) run at a flow rate of 0.6 ml h^{-1} in a Chromspher C18 column (Chrompack, Middelburg, The Netherlands).

Protein determination. Protein content in cell extracts was determined by a modified Lowry method (17).

Biomass concentration. Measurement of the optical density at 540 nm (OD_{540}) and estimation of the dry weight from the OD data were done as described previously (6).

Enzyme assays. Extracts of culture cells sampled from the fermentors were prepared by sonication in 100 mM phosphate buffer (pH 7.5) containing 10% acetone at 4°C and subsequent centrifugation at 40,000 × *g*. BADH and BZDH activities were determined by previously described methods (23), except that the benzaldehyde concentration in the BZDH assay was changed to 10 μM in order to minimize the reverse activity of BADH. Catechol 2,3-dioxygenase (C23O) and 2-hydroxymuconic-semialdehyde hydrolase (HMSH) activities were determined as described in references 20 and 21, respectively.

mRNA analysis. Samples of 8 ml from the fermentors were taken in precooled (−5°C) sample bottles and further cooled to 0°C in glass centrifuge tubes on a mixture of ice, water, and NaCl (−5°C) for 2 min. After centrifugation and disposal of the supernatant, the concentrated cell suspensions were transferred to a precooled Eppendorf tube (−5°C) and frozen at −70°C for storage. For analysis of the mRNA, total RNA was extracted by the hot guanidium isothiocyanate-phenol method slightly modified as described previously (14). The relative levels of each specific messenger were estimated by reverse primer extension of equal amounts of total RNA, using the following oligonucleotides: 5′-GGGTCGGTGAACATCTCG C-GCTTGC-3′ for Pm-dependent transcripts, 5′-GGCCAGC GTCACAGACTCCAGGCG-3′ for Pu, 5′-GAGACTGCAT AGGGCTCGGCGTGG-3′ for Ps, and 5′-ACGGATCTGGC TGCTAAGGTCTTGC-3′ for Pr.

The cDNA obtained in this way (134 nucleotides [nt] for Pu, 208 nt for Ps, and 127 nt for Pm) reflects the specific mRNA concentration in the sample. Primer extension analysis of RNA samples, denaturing polyacrylamide gel electrophoresis (PAGE), and estimation of cDNA relative amounts were carried out as previously described (14). The density of the bands in autoradiographs was scanned with a Perkin-Elmer densitometer which recorded the peak areas.

Induction experiments. Induction experiments were performed with steady-state continuous cultures grown on succinate-mineral medium. Noninduced enzyme levels were determined in extracts from cells sampled 10 to 30 min before the supply of an inducer. For the simultaneous induction of the upper and *meta*-cleavage pathway enzymes, at time zero *o*-xylene was supplied through the gaseous phase as described above, while the incoming fresh growth medium remained unchanged. For induction of only the *meta*-cleavage pathway, the chemostat culture fluid at time zero was supplied with 2,3-DMB to reach a final concentration of 1 mM and the succinate-mineral medium was replaced with a similar medium

containing in addition 1 mM 2,3-DMB, so that the concentration of the aromatic remained constant in time. At regular time intervals, samples were taken from the continuous cultures and subjected to the extraction procedure and enzyme assays.

Mathematical analysis of induction patterns of enzymes in chemostat-grown cells. Symbols used in equations 1 to 5 are defined as follows: E, enzyme level (milliunits per milligram of protein); E_0, noninduced enzyme level before supply of an inducer (milliunits per milligram of protein); E_{ss}, steady-state enzyme level (milliunits per milligram of protein); q_{enz}, synthesis rate of enzyme (milliunits per milligram of protein per hour); B, turnover rate of enzyme (per hour); D, dilution rate (per hour); and t_{lag}, lag time before full synthesis of the enzyme (hours).

In a continuous culture with a constant biomass concentration and a constant dilution rate, the differential rate of the expression of a certain enzyme in response to the supply of an inducer is dependent on the synthesis rate (q_{enz}), the dilution of the existing enzyme due to cell growth (equals the dilution rate D), and the turnover rate of the enzyme (B), according to the following equation (12):

$$dE/dt = q_{enz} - (D + B)E \quad (1)$$

If we assume that $E = E_0$ until t_{lag} and that q_{enz} and B are constant starting from t_{lag} throughout the experiment, integration with the boundary conditions $E(t = t_{lag}) = E_0$ and $E(t = \infty) = E_{ss}$ yields

$$E(t) = E_{ss} - (E_{ss} - E_0)e^{-q_{enz}(t - t_{lag})/E_{ss}} \quad (2)$$

and

$$B = q_{enz}/E_{ss} - D \quad (3)$$

Values for t_{lag}, E_{ss}, and q_{enz} were generated by nonlinear least-square fitting of equation 2 to the experimental data obtained by the change of E with time, assuming for E_0 the value determined for E at time zero. For the fitting procedure, the computer program used was ENZFITTER (Elsevier, Biosoft). It was set to weigh the datum points equally and to assume a proportional error distribution in the enzyme assays. Only the datum points with values for the enzyme activity E of at least 50% higher than determined at time zero were included in the fitting procedure. The program estimates the standard error of each of the generated parameters by the matrix inversion method.

Subsequently, the value for the turnover rate B was calculated according to equation 3. The relative standard error for B was estimated as the square root of the sum of the square numbers of the relative standard errors for the values for q_{enz} and E_0. The half-life of the enzyme ($t_{1/2}$) was calculated from the turnover rate B by the expression

$$t_{1/2} = \ln 2/B \quad (4)$$

Mathematical analysis of deinduction patterns. When one assumes that after elimination of the inducer, q_{enz} decreases instantaneously to noninduced levels, integration of equation 1 with the boundary conditions $E(t = 0) = E_{ss}$ and $E(t = \infty) = E_0$ yields

$$E(t) = E_0 - (E_0 - E_{ss})e^{-(D + B)t} \quad (5)$$

Values for B and E_0 were generated by fitting equation 5 to the experimental data for the change of E with time, entering for E_{ss} the empirical value for E at the starting point of deinduction (time zero). The fitting procedure is similar to that described above. All datum points were included.

RESULTS

Growth in fermentors. *P. putida*(pWW0) was grown in continuous culture in a mineral salts medium containing succinate as the sole source of carbon and energy. In separate fermentors, three different growth conditions were imposed on the strain: (i) succinate-limited growth at a dilution rate of 0.05 h^{-1}; (ii) succinate-limited growth at a dilution rate of 0.5 h^{-1}, and (iii) nonlimited growth on succinate in a phauxostat culture (μ = ca. 0.96 h^{-1}). Five days after inoculation of the fermentors, steady-state conditions were established. The OD_{540} of both succinate-limited cultures remained constant at about 0.60, and the OD_{540} of the phauxostat culture was about 0.85, corresponding to dry weights of about 330 and 470 mg $liter^{-1}$, respectively. The cultures were then supplemented with *o*-xylene (2.5 μM) or 2,3-DMB (1 mM). At regular intervals samples were taken from the culture and subjected to enzymatic assays (BADH, BZDH, C23O, and HMSH) and mRNA analysis.

Induction kinetics of BADH, BZDH, and C23O by *o*-xylene. After introduction of *o*-xylene in the chemostat, samples were periodically removed and the enzyme levels of BADH, BZDH, and C23O were determined. In the two succinate-limited cultures (D = 0.05 and 0.5 h^{-1}), the increase in activity of the upper pathway enzymes BADH and BZDH began almost immediately after the supply of *o*-xylene. The lag time before full synthesis of C23O (about 50 min) was longer than that observed for BADH and BZDH, especially at the highest dilution rate. The induction patterns for BADH and C23O are shown in Fig. 2. From the experimental data obtained in the assays done in Fig. 2 and other similar assays, the lag times, synthesis rates, steady-state levels, and turnover rates were calculated as described in Materials and Methods (Table 1).

No detectable levels of BADH and BZDH were found in cells grown at maximal rate under nonlimiting succinate growth conditions in the phauxostat culture after exposure to *o*-xylene. The level of C23O activity was measurable, but these levels were similar to those determined in the absence of *o*-xylene (Table 1). When metabolizable upper pathway substrates (30 to 40 μM toluene and 5 mM *m*-methylbenzylalcohol) were used instead of *o*-xylene, BADH activities were detectable but remained below 50 mU mg of $protein^{-1}$.

To assess whether the enzyme level determined in the chemostat reflected the transcriptional state of the corresponding promoters on the TOL plasmid, the mRNA levels of Pm, Pu, Ps, and Pr were measured. The XylR regulator stimulates transcription from the (σ^{54}-dependent) Pu and Ps promoters in response to the presence of *o*-xylene. XylR regulator is expressed constitutively (11), and XylR mRNA was synthesized under all growth conditions tested in this study (not shown). At the lowest dilution rate (0.05 h^{-1}) after the supply of *o*-xylene, the Pu and Ps transcript levels increased about 20-fold during the first 5 min, remaining relatively constant throughout the experiment thereafter (t = 15 min, 60 min, 5 h, and 24 h; Fig. 3). The Pm transcript, whose levels were not detectable at time zero, stabilized after 15 min, showing an intermediate level at t = 5 min (Fig. 3). This short delay in induction of the *meta*-cleavage pathway was observed previously when upper pathway effectors were used in batch culture (results not shown) and probably reflects the time required for the overproduction of the XylS protein responsible for activation of Pm. At D = 0.5 h^{-1} in the absence of *o*-xylene at time zero, the levels of Pu, Pm, or Ps transcripts were undetectable, while their steady-state levels in the presence of *o*-xylene were equal to or slightly lower than those measured under similar conditions at D = 0.05 h^{-1} (not shown). At maximal growth rate,

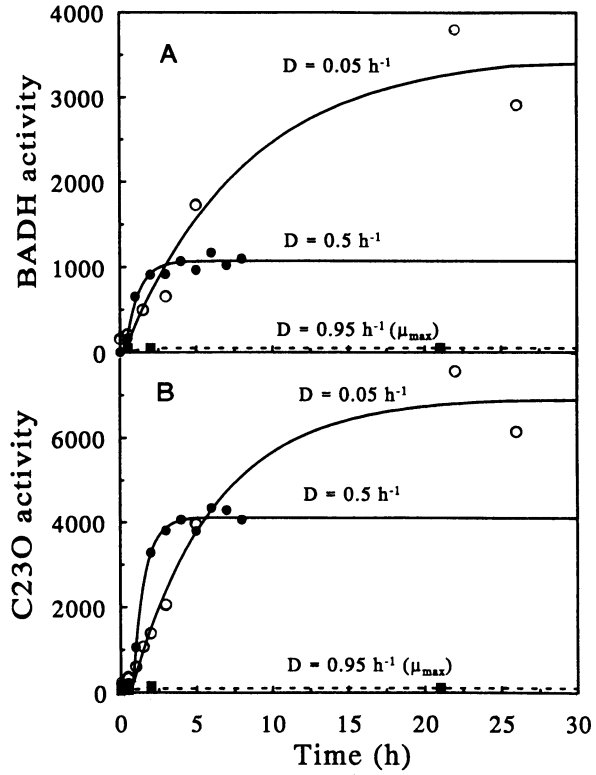

FIG. 2. Induction of BADH (A) and C23O (B) by *o*-xylene in *P. putida*(pWW0) grown in continuous culture under succinate limitation at a dilution rates of 0.05 h^{-1} (○) and 0.5 h^{-1} (●) or at nonlimited growth rate on succinate (■). Activities are expressed as milliunits per milligram of protein. Solid lines represent best-fit curves of equation 2 to the experimental values of the enzyme activities corresponding to the data in Table 1; broken lines represent best-fit curves of equation 2 to the experimental data obtained under nonlimiting growth conditions, assuming absence of enzyme turnover.

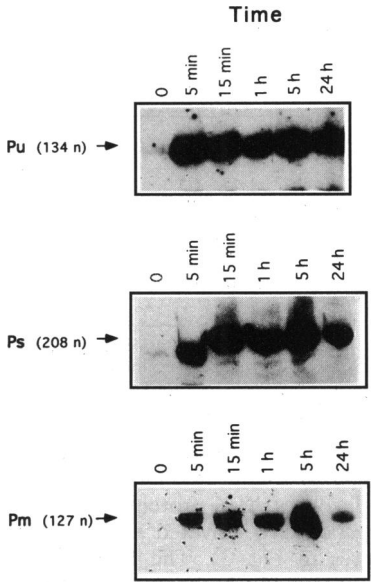

FIG. 3. Level of mRNA derived from Pu, Ps, and Pm promoters in response to *o*-xylene at $D = 0.05\ h^{-1}$. *P. putida*(pWW0) grown in continuous culture under succinate-limited conditions at $D = 0.05\ h^{-1}$ was induced with *o*-xylene, samples were collected at the indicated times, and total RNA was extracted. The presence of messengers was analyzed by reverse primer extension of 20 μg of total RNA with the corresponding labelled specific oligonucleotides. The figure shows urea-PAGE of the cDNA corresponding to transcripts derived from Pu (134 nt [n]), Ps (208 nt), and Pm (127 nt).

the Pu, Pm, and Ps transcripts were undetectable in both the absence and presence of *o*-xylene. These results confirm that the different enzyme levels of the upper and *meta*-cleavage pathway enzymes under C-limited and nonlimited culture conditions reflect the transcriptional status of the TOL promoters.

Induction kinetics of C23O and HMSH by 2,3-DMB. The supply of 2,3-DMB to the succinate-limited cultures at both dilution rates resulted in a rapid increase in the Pm transcript levels and in levels of the *meta*-cleavage enzymes C23O and HMSH.

The lag times before full synthesis of C23O and HMSH were less than 15 min (Table 2; Fig. 4), and the Pm transcript level stabilized within 5 min (not shown). Figure 5 shows that at both dilution rates, the Pm transcript level was nondetectable in the absence of 2,3-DMB, and no significant difference between $D = 0.05\ h^{-1}$ and $D = 0.5\ h^{-1}$ in the presence of 2,3-DMB was observed (note difference in total RNA amounts loaded per lane [15 μg for $D = 0.05\ h^{-1}$ and 50 μg for $D = 0.5\ h^{-1}$]). During nonlimited growth on succinate in the presence of 2,3-DMB, the C23O level was 93% lower (600 mU mg of protein^{-1}) than in the succinate-limited cultures.

Induction kinetics of C23O and HMSH by both 2,3-DMB and *o*-xylene. As stated previously, there are two ways of activating the Pm promoter. One involves the low basal levels

TABLE 1. Induction of TOL pathway enzymes by *o*-xylene in *P. putida*(pWW0) grown on succinate-mineral medium in continuous culture[a]

Growth condition	Enzyme	Lag time[b] (min)	Synthesis rate (mU mg of protein^{-1} h^{-1})	Steady-state level (mU mg of protein^{-1})	Turnover rate (h^{-1})
Succinate limited ($D = 0.05\ h^{-1}$)	BADH	34 ± 36	432 ± 165	3,530 ± 460	0.072 ± 0.049
	BZDH	−4 ± 15	157 ± 138	446 ± 24	0.30 ± 0.09
	C23O	50 ± 23	1,248 ± 323	6,960 ± 470	0.13 ± 0.05
Succinate limited ($D = 0.5\ h^{-1}$)	BADH	20 ± 6	1,240 ± 250	1,074 ± 35	0.65 ± 0.24
	BZDH	8 ± 14	410 ± 130	502 ± 31	0.32 ± 0.26
	C23O	48 ± 3	5,230 ± 630	4,120 ± 87	0.77 ± 0.15
Nonlimited ($D = 0.95\ h^{-1}$)	BADH	ND	ND	<20	ND
	BZDH	ND	ND	<10	ND
	C23O	ND	ND	107 ± 34	ND

[a] Values are the means ± standard errors expected from nonlinear regression analysis of the experimental data. ND, not done.
[b] *o*-Xylene was supplied at time zero; synthesis rate of enzyme was maximal after the indicated time period.

TABLE 2. Induction of *meta*-cleavage pathway enzymes by 2,3-DMB in *P. putida*(pWW0) grown on succinate-mineral medium in continuous culture[a]

Growth condition	Enzyme	Lag time[b] (min)	Synthesis rate (mU mg of protein^{-1} h^{-1})	Steady-state level (mU mg of protein^{-1})	Turnover rate (h^{-1})
Succinate limited (D = 0.05 h^{-1})	C23O	1.6 ± 4.4	1,700 ± 130	9,120 ± 390	0.14 ± 0.02
	HMSH	3.0 ± 3.3	490 ± 70	1,110 ± 40	0.40 ± 0.06
Succinate limited (D = 0.5 h^{-1})	C23O	1.1 ± 1.4	4,180 ± 480	9,520 ± 1,030	−0.06 ± 0.07
	HMSH	13.7 ± 0.8	1,440 ± 190	1,740 ± 120	0.33 ± 0.13
Nonlimited (D = 0.97 h^{-1})	C23O	ND	ND	600 ± 200	ND

[a] Values are the means ± standard errors expected from nonlinear regression analysis of the experimental data. ND, not done.
[b] 2,3-DMB was supplied at time zero; synthesis rate of enzyme was maximal after the indicated time period.

of XylS protein that become activated in the presence of benzoates (2,3-DMB-induced cultures; Fig. 5); The other involves high levels of XylS protein (not benzoate activated) produced in response to activated XylR protein (see *o*-xylene-induced cultures in Fig. 3). The following experiment was designed to determined whether transcription levels from Pm were saturated in any of those two conditions.

A steady-state succinate-limited culture in which the *meta*-cleavage pathway had been induced by 2,3-DMB was supplied with *o*-xylene at time zero. The C23O levels subsequently increased gradually from 9,000 to 19,000 mU mg of protein^{-1} while the BADH levels increased from a level below 100 to 1,150 mU mg of protein^{-1} (Fig. 6).

The Pm RNA amount, which was present at the expected level at time zero as a result of the induction by 2,3-DMB, significantly increased during the first 15 min after the addition of *o*-xylene and stabilized thereafter (Fig. 7). The Ps transcript levels increased 30-fold during the first 5 min after the addition of *o*-xylene (Fig. 7).

Catabolite repression by carbon and energy sources other than succinate. Phauxostat cultures were also run with lactate or pyruvate as the sole source of carbon and energy. The nonlimited growth rates on these compounds were 22 and 24% lower than on succinate (0.75 and 0.71 h^{-1}, respectively). The BADH levels in response to 2.5 μM *o*-xylene were nondetectable for lactate and 110 mU mg of protein^{-1} for pyruvate.

Deinduction experiments. To obtain an indication of the reliability of the values for enzyme turnover rates derived from the induction experiments, we also determined the disappearance rate of enzyme activity after removal of the inducer. Succinate-limited cultures (D = 0.05 and 0.5 h^{-1}) were grown to steady state in the presence of 2,3-DMB. To reduce the synthesis rates of the *meta*-cleavage pathway enzymes C23O and HMSH to basal levels, the 2,3-DMB was removed by washing the cells. Subsequently, the cells were grown further in continuous culture under similar conditions in the absence of 2,3-DMB. The resulting gradual decrease in the activities of C23O and HMSH was mathematically analyzed by using equation 5 (Fig. 8). The values for the turnover rates generated by this procedure are shown in Table 3, which also includes the

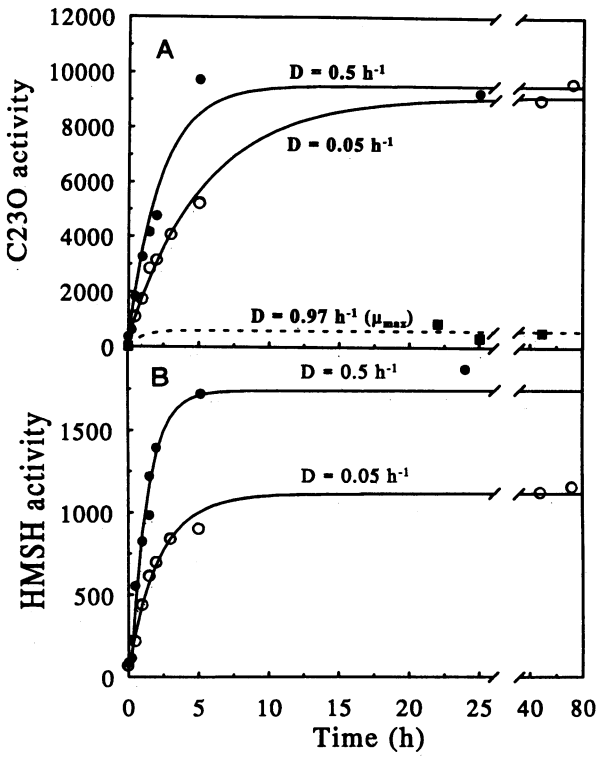

FIG. 4. Induction of C23O (A) and HMSH (B) by 2,3-DMB in *P. putida*(pWW0) grown in continuous culture under succinate limitation at a dilution rate of either 0.05 h^{-1} (○) or 0.5 h^{-1} (●) or at nonlimited growth rate on succinate (■). Activities are expressed as milliunits per milligram of protein. Solid lines represent best-fit curves of equation 2 to the experimental values of the enzyme activities corresponding to the data in Table 2; the broken line represents a best-fit curve of equation 2 to the experimental data obtained under nonlimiting growth conditions, assuming absence of enzyme turnover.

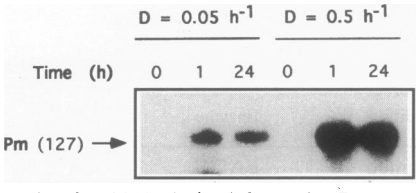

FIG. 5. Levels of mRNA derived from the Pm promoter in response to 2,3-DMB at different growth rates. *P. putida*(pWW0) grown in continuous culture under succinate-limited conditions at D = 0.05 or 0.5 h^{-1} was induced with 2,3-DMB. Samples were collected at the indicated times, and total RNA was extracted. The presence of messenger derived from Pm was analyzed by reverse primer extension of 15 μg (D = 0.05 h^{-1}) or 50 μg (D = 0.5 h^{-1}) of total RNA with the corresponding labelled specific oligonucleotides. The figure shows urea-PAGE of the cDNA corresponding to transcripts derived from Pm (127 nt).

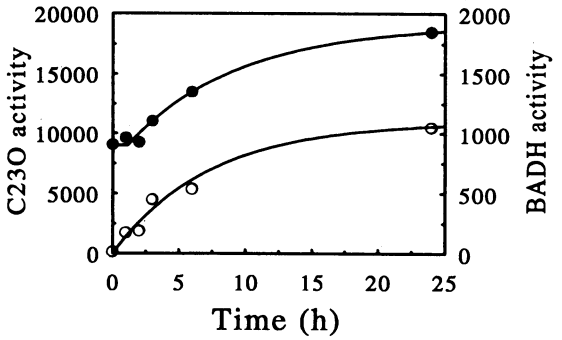

FIG. 6. Induction of C23O (●) and BADH (○) by *o*-xylene in cells of *P. putida*(pWW0) grown in continuous culture under succinate limitation in the presence of 2,3-DMB at a dilution rate of 0.05 h^{-1} (activities are expressed as milliunits per milligram of protein). Solid lines represent best-fit curves of equation 2 to the experimental data obtained.

values for the turnover rates derived from the induction experiments.

DISCUSSION

We have compared the induction kinetics of the TOL catabolic genes in cells of *P. putida*(pWW0) growing on succinate either under carbon and energy limitation or at a nonlimited growth rate. The transcript levels of Pu, Pm, and Ps, as well as the levels of two enzymes of the *meta*-cleavage pathway (C23O and HMSH) and two enzymes of the upper pathway (BADH and BZDH), were determined. The kinetics of enzyme induction in response to the nonmetabolizable inducers

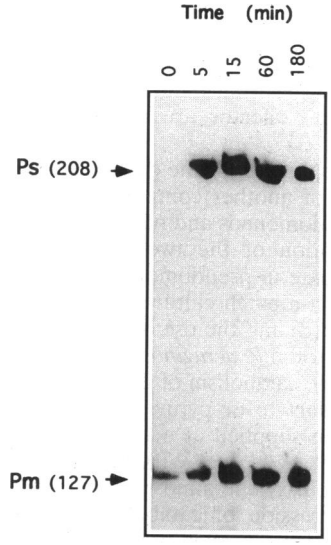

FIG. 7. Levels of mRNA derived from Ps and Pm promoters in response to the addition of *o*-xylene to cells growing at $D = 0.05\ h^{-1}$ in the presence of 2,3-DMB. *P. putida*(pWW0) was grown in continuous culture under succinate-limited conditions at $D = 0.05\ h^{-1}$ in the presence of 2,3-DMB. At time zero, *o*-xylene was added, and samples were collected thereafter at the indicated times for total RNA extraction. The presence of messengers was analyzed by reverse primer extension of 25 μg of total RNA with the corresponding labelled specific oligonucleotides. The figure shows urea-PAGE of the cDNA corresponding to transcripts derived from Ps (208 nt) and Pm (127 nt).

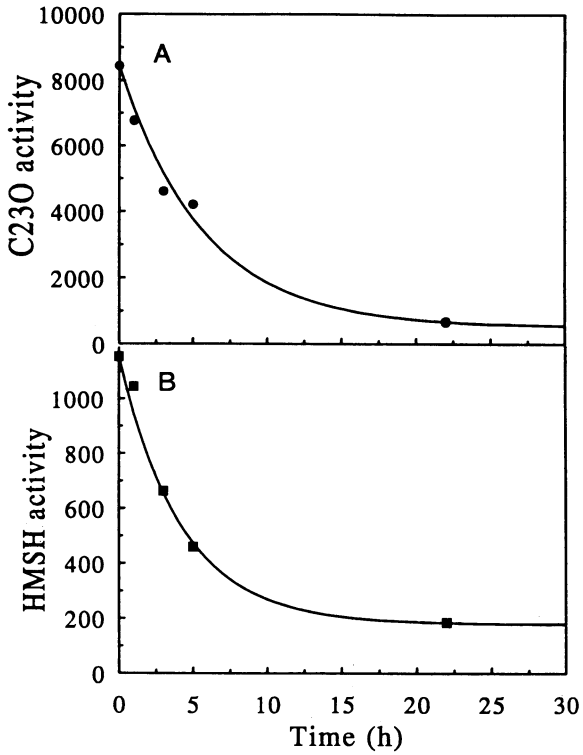

FIG. 8. Deinduction of C23O (A) and HMSH (B) after the removal of 2,3-DMB from a culture of cells of *P. putida*(pWW0) grown in continuous culture under succinate limitation at a dilution rate of 0.05 h^{-1} (activities are expressed as milliunits per milligram of protein). Solid lines represent best-fit curves of equation 5 to the experimental values of the enzyme activities, generating estimates for the turnover rates of the enzymes as included in Table 3.

2,3-DMB, *o*-xylene, and 2,3-DMB plus *o*-xylene were analyzed. This was done by using a model that describes mathematically how the steady-state levels of an enzyme are the result of its rates of synthesis and turnover and the dilution rate.

Continuous cultivation techniques allowed growth of cells in a constant physiological state with respect to the cells' carbon and energy status (8), while the use of nonmetabolizable inducers, rather than substrates of the pathway, allowed us both to study induction kinetics without altering the carbon and energy status of the cells and to maintain a constant concentration of the effector. Our results revealed that the mRNA transcripts of Pu, Ps, and Pm in response to *o*-xylene and of Pm in response to 2,3-DMB reached their maximum levels shortly after (5 min) the addition of the nonmetaboliz-

TABLE 3. Turnover rates of C23O and HMSH as derived from 2,3-DMB induction assays and deinduction assays on succinate-limited continuous cultures ($D = 0.05\ h^{-1}$)[a]

Enzyme	Induction assays		Deinduction assays	
	Turnover rate (h^{-1})	Half-life[b] (h)	Turnover rate (h^{-1})	Half-life (h)
C23O	0.14 ± 0.02	5.1	0.13 ± 0.02	5.4
HMSH	0.40 ± 0.06	1.8	0.19 ± 0.02	3.7

[a] Values are the means ± standard errors expected from nonlinear regression analysis of the experimental data.
[b] Calculated from the turnover rates, using equation 4.

able inducer to succinate-limited cultures and remained constant throughout the experiment (24 h) (Fig. 3 and 5). This finding suggests that the synthesis rates of the corresponding enzymes remained constant with time at a fixed growth rate, allowing the application of the model presented for the mathematical analysis of the induction patterns. The model assumes that under steady-state conditions, the synthesis rate of an enzyme is counterbalanced by the rate of its turnover and the dilution rate. Parameters derived from applying the model to the induction experiments included the synthesis rate, steady-state level, and turnover rate of the enzyme.

Under succinate-limiting growth conditions, only gradual differences in the parameters analyzed were found. In general, the synthesis rates of the four enzymes tested were approximately three times higher at the high dilution rate (0.5 h^{-1}) than at the low dilution rate (0.05 h^{-1}). The higher synthesis rates at the higher growth rate cannot be explained merely on the basis of the corresponding mRNA levels, since in cultures supplemented with 2,3-DMB, no differences were found between the Pm mRNA level in cells grown at $D = 0.05\ h^{-1}$ and the level in cells growing at $D = 0.5\ h^{-1}$. The level of Pu mRNA transcript in succinate-limited cells growing in the presence of *o*-xylene was lower at the high growth rate ($D = 0.5\ h^{-1}$) than at the low growth rate. A relatively high rRNA content and a high carbon and energy status known to exist in cells grown at high dilution rates (8) may be responsible for the observed higher translation rates at the higher growth rate. The steady-state levels of all examined enzymes differed by a factor of less than 2 (comparing the two dilution rates), except for BADH, which showed a steady-state level three times higher at the low dilution rate than at the higher one. Mathematical analysis of the induction pattern indicated that this could be attributed to a relatively low turnover rate of BADH at a dilution rate of 0.05 h^{-1} rather than to a higher synthesis rate.

Under nonlimiting growth conditions (the surplus of succinate feed allowed a constant residual concentration of 4 mM succinate in the culture), no induction of the upper pathway in response to *o*-xylene could be detected at either the transcriptional level (Pu, Ps) or the enzyme activity level. The regulatory XylR protein is expressed constitutively from two tandem promoters (11), and previous work (data not shown) has shown that *xylR* mRNA levels are influenced relatively little by the growth conditions. We have confirmed this observation in this study. Therefore, we assume that the suppression of upper pathway induction is wholly due to the inability of the XylR-effector complex to stimulate transcription from the Pu promoter. The full repression of the upper pathway promoter and the Ps promoter was contrasted with the partial (93%) repression of the *meta*-cleavage pathway with 2,3-DMB as an inducer under similar conditions. In this case, the low induction level from Pm correlates with the very low levels of the *xylS* mRNA transcript detected. These levels are much lower than under succinate-limited conditions (Fig. 9). This finding suggests that both induced and basal expression from Ps are subject to catabolite repression. Here it is worth noting that in footprinting studies with the similar σ^{54}-dependent Pu promoter, XylR protein has been shown as always bound to the promoter, independent of the presence of an effector (1). If this were also the case for the Ps promoter, the conclusion would be that this binding promotes a basal expression that is also subject to catabolite repression.

We do not know whether the observed low levels of XylS protein can account fully for the modest inducing effect of 2,3-DMB when succinate is present at nonlimiting concentrations. A study by Hugouviex-Cotte-Pattat et al. (10) indicated that when XylS levels are kept constant, expression of the Pm promoter is lower in the exponential growth phase (nonlimited growth) than in the stationary phase (comparable to nutrient-limited growth in chemostat). However, these experiments were performed with an *Escherichia coli* strain harboring a Pm-*lacZ* fusion, and it is uncertain to what extent this effect may also play a role in *P. putida*(pWW0).

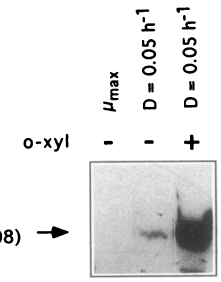

FIG. 9. *xylS* mRNA transcript levels under succinate-limited and nonlimited growth conditions. Samples were collected from steady-state cultures of *P. putida*(pWW0) grown under succinate-limited ($D = 0.05\ h^{-1}$) or nonlimited conditions in the presence (+) or absence (−) of *o*-xylene (o-xyl), and total RNA was extracted. The presence of messengers derived from the Ps promoter was analyzed by reverse primer extension of 50 μg ($D = 0.05\ h^{-1}$) of total RNA with the corresponding labelled specific oligonucleotide. The figure shows urea-PAGE of the cDNA corresponding to transcripts derived from Ps (208 nt).

An interesting finding was that the supply of *o*-xylene to a succinate-limited and 2,3-DMB-supplemented chemostat culture led to a strong increase in the expression of the *meta*-cleavage pathway (Fig. 6 and 7). This finding suggests that the basal levels of the XylS protein at a low dilution rate in the presence of an effector were not saturating the Pm promoter. Since the steady-state levels of C23O (19,000 mU mg of protein^{-1}) were also higher than its steady-state levels in the presence of *o*-xylene alone (7,000 mU mg of protein^{-1}), we suggest that induced levels of XylS protein in the absence of an effector are not saturating for the Pm promoter either.

In fact, the suppression of the catabolism of one compound by the presence of another compound is a well-known phenomenon in pseudomonads and results in batch cultures in the sequential utilization of the two carbon sources (diauxie). Examples of diauxie in pseudomonads are the use of acetate prior to oxalate in a batch culture of *P. oxalaticus* containing both compounds (8) and the use of succinate in preference to glucose or mannitol in *P. aeruginosa* (13). In the same study, it was shown that the catabolism of glucose was not repressed by the presence of lactate or pyruvate (13). In our study, both compounds, when supplied at a nonlimited rate to a phauxostat culture of *P. putida*(pWW0), repressed expression from Pu in response to *o*-xylene almost completely.

Catabolite repression of catabolic pathways for aromatic compounds has been observed previously for protocatechuate dioxygenase in *Pseudomonas cepacia* (24). Catabolite repression was also observed for the degradation pathway of aniline in *Pseudomonas multivorans* An1. This strain was shown to utilize carbohydrates, organic acids, and amino acids in preference to aniline in mixed-substrate cultures (9). Another aniline-degrading pseudomonad, however, was shown to utilize aniline and other organic substrates simultaneously (9). In other studies, it also became apparent that catabolite repression of catabolic pathways for xenobiotic compounds is not as

common as could be expected; 3-phenoxybenzoate metabolism by a strain of *Pseudomonas delafieldii* was shown to be nonsusceptible to catabolite repression (22), as was the degradation of 3-chloroaniline by *Pseudomonas acidovorans* (7). Release of catabolite repression of catabolic enzymes in a chemostat culture limited by a potentially repressing substrate, as observed in this study, is a generally occurring phenomenon (8, 16). A diminished catabolite repression under carbon-limited conditions was reasoned to be of possible advantage because it allows the concurrent utilization of a mixture of (growth-limiting) substrates (16). This consideration is in agreement with our observation that a nonlimiting concentration of succinate represses induction of the TOL pathway by *o*-xylene under nitrogen limitation, even when the dilution rate is as low as 0.05 h^{-1} (results not shown). This also indicates that the carbon and energy status of the cell, rather than the growth rate, determines the occurrence of catabolite repression of the TOL pathway.

The mathematical analysis of the induction experiments allowed estimation of the turnover rates of individual enzyme activities. From the induction kinetics by 2,3-DMB at the lowest dilution rate (0.05 h^{-1}), it was deduced that C23O exhibited a half-life of about 5 h and that it was more stable than HMSH, whose half-life was estimated to be about 2 h. The reasonable agreement of the half-life data with the data derived from the deinduction experiments (Table 3) suggests that the synthesis rate of the enzymes after the supply of 2,3-DMB is indeed constant. At the high dilution rate (0.5 h^{-1}), the half-lives for C23O and HMSH were relatively long; however, these estimates are less accurate because the contribution of enzyme turnover to the enzyme activity loss was small compared with the contribution of cell growth (dilution of the enzyme). The half-life values for BZDH derived from the induction experiments at a $D = 0.05$ and 0.5 h^{-1} were both ca. 2 h. BADH was found to be very stable at the lowest dilution rate ($t_{1/2} = 10$ h) but rather unstable at the highest dilution rate ($t_{1/2} = 1$ h). The turnover rate of key enzymes of catabolic pathways may have influence on biodegradation kinetics in natural environments and may be best studied in low-dilution-rate chemostat cultures in which the natural conditions for a low growth rate and a limited supply of nutrients are mimicked.

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