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Catabolite Repression of the Toluene Degradation Pathway in *Pseudomonas putida* Harboring pWW0 under Various Conditions of Nutrient Limitation in Chemostat Culture

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In earlier studies, the pathway of toluene and *m*- and *p*-xylene degradation (TOL pathway) in *Pseudomonas putida* (pWW0) was found to be subject to catabolite repression when the strain was grown at the maximal rate on glucose or succinate in the presence of an inducer. This report describes catabolite repression of the TOL pathway by succinate in chemostat cultures run at a low dilution rate ($D = 0.05\ h^{-1}$) under different conditions of inorganic-nutrient limitation. The activity of benzylalcohol dehydrogenase (BADH) in cell extracts was used as a measure of the expression of the TOL upper pathway. When cells were grown in the presence of 10 to 15 mM succinate under conditions of phosphate or sulfate limitation, the BADH activity in response to the nonmetabolizable inducer *o*-xylene was less than 2% of that of cells grown under conditions of succinate limitation. Less repression was found under conditions of ammonium or oxygen limitation (2 to 10% and 20 to 35%, respectively, of the BADH levels under succinate limitation). The BADH expression levels determined under the different growth conditions appeared to correlate well with the mRNA transcript levels from the upper pathway promoter (Pu), which indicates that repression was due to a blockage at the transcriptional level. The *meta*-cleavage pathway was found to be less susceptible to catabolite repression. The results obtained suggest that the occurrence of catabolite repression is related to a high-energy status of the cells rather than to a high growth rate or directly to the presence of growth-saturating concentrations of a primary carbon and energy source.

The observation that many of the xenobiotic compounds investigated to date are biodegradable in laboratory bacterial cultures (30) is, with respect to their environmental fate, not as reassuring as is often suggested. The actual biodegradation rates of aromatics under natural conditions may be very low, frequently because of insufficient concentrations of oxygen (4, 6) or a limited bioavailability of the contaminant as a result of binding to soil particles or low water solubility (23).

An additional factor contributing to low degradation rates under field conditions may be that the bacterial subpopulation capable of degrading a certain pollutant does not fully express its catabolic potential as a result of the presence of other, more readily degradable, substrates. This phenomenon, which results in batch culture in the sequential utilization of the carbon sources (diauxie), is generally referred to as catabolite repression (18). The expression of catabolic pathways for camphor (11), protocatechuate (35), benzene (22), styrene (25), and aniline (13) was reported earlier to be subject to catabolite repression. The most detailed studies on aromatic compounds have been performed on *Pseudomonas putida* mt-2 harboring the TOL plasmid pWW0 that encodes the degradation of toluene, *m*- and *p*-xylene, pseudocumene, and *m*-ethyltoluene (3) (Fig. 1). Hugouvieux-Cotte-Pattat et al. (15) found a long delay in the induction of the TOL pathway by methylbenzylalcohol when the strain was growing in batch culture on Luria-Bertani broth. This effect could be attributed to the amino acid content of the medium (19). In the former study (15), glucose was found to have a similar but less profound effect, which was later confirmed and further elaborated upon by Holtel et al. (14). In continuous culture, which allows the cultivation of cells for many generations under constant environmental conditions, a strong repression was found during nonlimited growth on succinate in the presence of an upper pathway inducer (7).

From the environmental point of view, these results are not particularly relevant since maximal growth rate conditions do not, in general, occur under field conditions. In soil and surface water, the degradation of xenobiotics is often limited by the available concentration of oxygen (4, 6), phosphate (16, 24, 28), or nitrogen (2, 16). We mimicked these limiting conditions in chemostat cultures of *P. putida* (pWW0) growing on a succinate-mineral medium and studied the inducibility of the TOL pathway in response to the nonmetabolizable inducer *o*-xylene. The inducibility of the TOL pathway was shown to be very low under conditions of phosphate or sulfate limitation and relatively high under conditions of oxygen or carbon and energy limitation. Ammonium-limiting conditions resulted in intermediate expression levels.

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).

Media. The growth medium was a mineral salts medium described previously (8) and supplemented with (i) 10 mM succinate when supplied to a carbon- and energy-limited culture (C limitation) or (ii) 20 mM for other conditions of nutrient limitation. For conditions of ammonium limitation (N limitation), the

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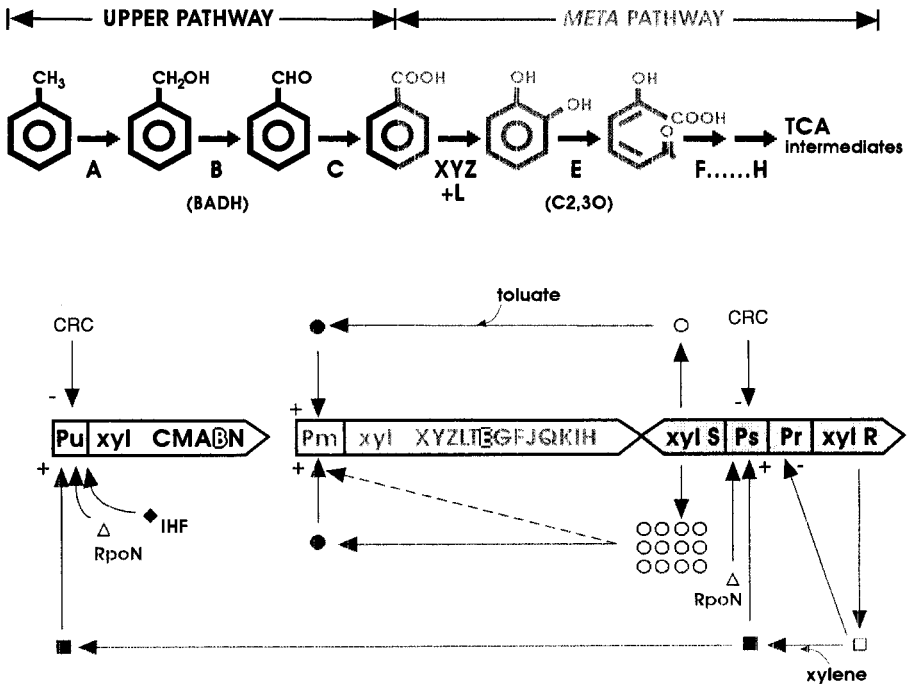

FIG. 1. First enzymatic steps and genetic organization of the TOL plasmid pWW0 upper and *meta*-cleavage pathways. Symbols: open square, inactive form of the XylR regulatory protein: closed square, activated XylR form that, with RpoN (open triangle), stimulates transcription from Ps, and with RpoN and integration host factor stimulates transcription from Pu (XylR by itself represses its own synthesis); open circle, nonactivated XylS regulatory protein, which stimulates transcription from Pm only when present at a high concentration (dotted arrow); closed circle, active XylS protein that stimulates transcription from Pm also when present at a low concentration; +, stimulation of transcription; −, inhibition of transcription (modified from references 20 and 29). Abbreviations: IHF, integration host factor; CRC, catabolite repression control; TCA, tricarboxylic acid.

NH_4Cl concentration was 1.88 mM; for conditions of phosphate limitation (P limitation), the KH_2PO_4 concentration was 60 μM; and for conditions of sulfate limitation (S limitation), the Na_2SO_4 concentration was 15 μM. For conditions of oxygen limitation (O_2 limitation), the mineral salts concentrations were identical to the medium used for C limitation. Except for P limitation, the pH of chemostat cultures was maintained at 6.8 to 7.2 with media containing a 50 mM KH_2PO_4-K_2HPO_4 buffer set at pH 6.4 (conversion of 10 mM succinate under steady-state conditions led to an increase in pH of the growth medium to 7.0 ± 0.2). Silicone antifoaming agent (BDH) was added to the growth medium at a concentration of 62 μl $liter^{-1}$.

Chemostat culture. The fermentors used were custom made; the culture vessel was shaped like an Erlenmeyer flask (base diameter, 60 mm; top diameter, 34 mm) with a total volume of 125 ml and a working volume of 100 ml. The fermentor was equipped with two stainless-steel baffles. The pH was maintained at 7.0 under P limitation by automatic addition of NaOH (0.5 M). The cultures were stirred magnetically at 500 rpm with a Teflon-coated stirring bar (40 by 9 mm). Air was supplied at 100 ml min^{-1}, except for O_2 limitation, where the stirring speed was 200 rpm, and the incoming gas flow (33 ml min^{-1}) was a mixture of air and nitrogen (1:5). The temperature was maintained at 28 ± 1°C. After the cultures had achieved a steady state, the nonmetabolizable inducer of the TOL pathway *o*-xylene was supplied to the cultures through the gas phase by use of an additional airflow (33 ml h^{-1}) containing 10 to 50 μmol of *o*-xylene $liter^{-1}$. For O_2-limited cultures, nitrogen instead of air was used for this additional gas flow.

Analysis of *o*-xylene and succinate. Samples taken from a fermentor were filtered through a 0.22-μm-pore-size filter. For *o*-xylene analysis, samples of 10 μl were injected in a high-performance liquid chromatography apparatus equipped with a variable-wavelength detector (Hewlett-Packard 1050 series) set at 195 nm. The solvent was acetonitrile-water (86:14 [vol/vol]) run at a flow rate of 0.4 ml min^{-1} in a 10-cm Hypersil C_{18} column (Chrompack, Middelburg, The Netherlands). For succinate analysis, samples of 1 μl were analyzed with the same equipment with a 20-cm Chromspher C_{18} column from the same supplier and 5 mM H_2SO_4 in water as a solvent. Detection of succinate was at 207 nm.

Protein determination. Protein content in cell extracts was determined by a modification of the Lowry method (26).

Biomass concentration. The optical density at 540 nm (OD_{540}) of chemostat cultures was measured regularly. The dry weight of samples was measured under all conditions of limitations as described previously (9). No important influence of the growth condition was found for the relation between the data for the dry weight and OD_{540} values. An OD_{540} of 1.0 correlated with an average dry weight of 380 mg $liter^{-1}$. This value was then used to estimate the dry weight from the OD_{540} data.

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*. Benzyl alcohol dehydrogenase (BADH) and catechol 2,3-dioxygenase (C23O) activities were determined as described in references 34 and 31, respectively.

mRNA analysis. Samples were taken from chemostat cultures, immediately cooled, centrifuged, and frozen as described previously (7). Extraction and analysis of mRNA by reverse primer extension were done as described previously (21), with the oligonucleotide 5′-GGCCAGCGTCACAGACTCCAGGCG-3′ (hybridizing to the coding region of the first open reading frame of the upper pathway operon) to quantitate the relative amounts of transcripts; the intensities of the bands in autoradiographs were scanned with a Perkin-Elmer densitometer to determine the peak areas.

RESULTS

Growth in chemostats. The toluene- and *m*- and *p*-xylene-degrading strain *P. putida*(pWW0) was grown in a chemostat culture at a dilution rate of 0.05 h^{-1} (corresponding to a growth rate of 0.05 h^{-1} and a generation time of 14 h). Under all conditions of limitation applied (C, N, P, S, and O_2), steady-state conditions were established within 1 week. The OD_{540} and the residual concentrations of succinate showed variations from day to day of less than 10%. The average residual succinate concentrations, dry weights, and growth yields are shown in Table 1. The yields on succinate under P and N limitation were about two times lower than those under C and O_2 limitation. S-limited cultures showed intermediate values for the growth yield (Table 1).

Induction of the upper pathway of the TOL pathway by *o*-xylene. For each condition of limitation, three identical steady-state cultures, run in parallel, were supplied with three different concentrations of the nonmetabolizable inducer *o*-

TABLE 1. Growth characteristics of steady-state chemostat cultures (D = 0.05 h^{-1}) of *P. putida*(pWW0) growing under different conditions of limitation with succinate as the sole carbon and energy source in the presence of different concentrations of *o*-xylene

Limitation	[*o*-Xylene] (μM)	[Succinate] (mM)	OD_{540}	Dry wt (mg $liter^{-1}$)	Yield (mg of C [dry wt] mg of C^{-1})
C	0	0	0.60	227	0.47
	2.5	0	0.60	227	0.47
	6.4	0	0.56	212	0.44
	21	0	0.53	200	0.42
N	0	8.0	0.40	151	0.26
	2.6	8.2	0.41	155	0.27
	9.2	6.3	0.35	132	0.20
	16	7.5	0.50	189	0.32
P	0	7.0	0.42	158	0.25
	2.2	9.6	0.30	113	0.23
	6.1	6.0	0.34	129	0.19
	17.2	7.8	0.35	132	0.23
S	0	11.6	0.34	129	0.32
	1.8	10.4	0.36	136	0.30
	6.7	10.7	0.40	151	0.34
	17	11.6	0.35	132	0.33
O_2	0	12.0	0.43	162	0.42
	4.6	12.6	0.30	113	0.32
	7.1	15.5	0.26	98	0.46

xylene. Just before and 2 days after the supply of *o*-xylene, samples were taken from the chemostats. The enzyme level of BADH was determined immediately, and cells were frozen for later mRNA analysis. Prior to the introduction of *o*-xylene to the cultures, the BADH levels were found to be nondetectable (lower than 5 mU mg of $protein^{-1}$) for all conditions of limitation except C limitation (150 mU mg of $protein^{-1}$). Two days after the introduction of *o*-xylene, the BADH activity of all three C-limited cultures was expressed at maximal levels at the three *o*-xylene concentrations supplied (3, 6, and 21 μM). The BADH levels in the P- and S-limited cultures were all lower than 2% of the BADH levels in cells growing under C limitation (Fig. 2). The BADH levels under N limitation were less repressed (2 to 10% of the levels under C limitation, depending on the *o*-xylene concentration supplied). Under O_2 limitation, the repressing effect of growth-saturating concentrations of succinate was much smaller than that under P, S, or N limitation; at a *o*-xylene concentration of 5 or 7 μM, the BADH levels were 20 to 35% of the levels measured under C limitation (Fig. 2).

Analysis of Pu mRNA transcript levels. It was previously shown that repression of BADH expression levels during nonlimited growth on succinate in continuous culture was exerted at the transcriptional level (7). The transcription of mRNA from the upper pathway promoter Pu was analyzed under the different conditions of limitation described above. No Pu transcript was detected in the absence of an inducer under any of the limiting conditions tested. The maximum expression level was obtained in C-limited cultures in the presence of *o*-xylene (Fig. 3), which correlated with the high levels of BADH found in the same cultures. No Pu transcript was detected under either P limitation (Fig. 3) or S limitation (data not shown) in the presence of *o*-xylene, thus suggesting that the absence of BADH activity in these cultures was due to a total repression of transcription from the Pu promoter. In N-limited cultures, the presence of *o*-xylene allowed induction from the Pu promoter, although the level of expression was four times lower than that under C limitation. As for BADH activity, the repressing effect of excess succinate was the lowest under O_2 limitation, whereas mRNA levels were as high as 50% of the maximum levels obtained under C limitation (Fig. 3).

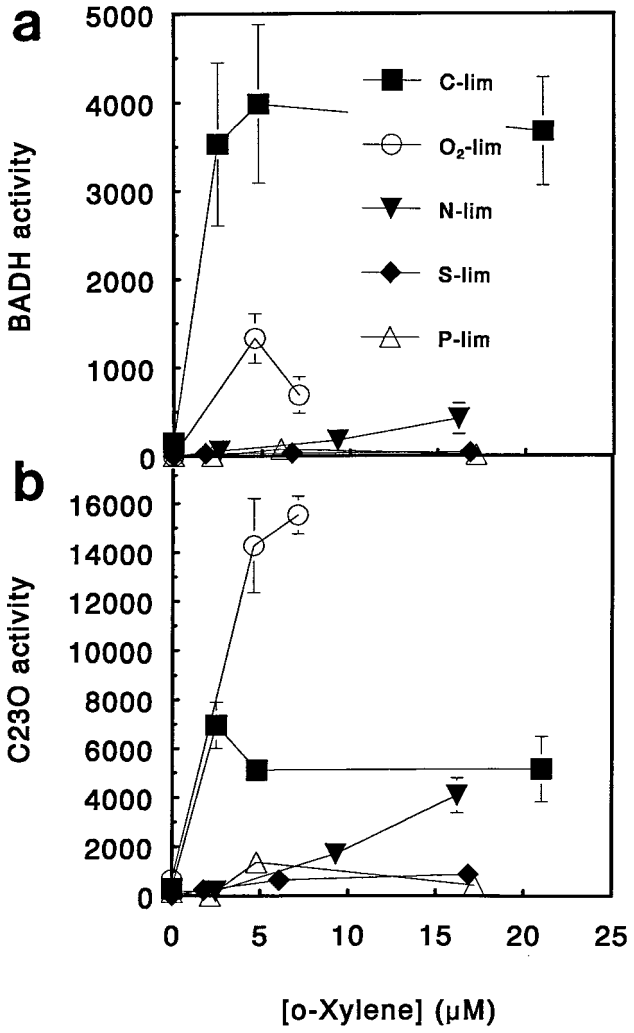

FIG. 2. Steady-state levels of BADH (a) and C23O (b) activities in response to different concentrations of the nonmetabolizable inducer *o*-xylene under different conditions of limitation (lim) (the carbon and energy source is succinate; the dilution rate is 0.05 h^{-1}). Activities are expressed as milliunits per milligram of protein. The errors bars indicate double standard errors of the mean and are shown only when larger than the size of the marker of the datum point.

Induction of the *meta* pathway by *o*-xylene. The specific activity of C23O was used as a measure of the induction level of the *meta* cleavage pathway. The C23O levels were repressed the most under P and S limitation (2 to 20% of the C23O levels under C limitation). Under N limitation, considerable catabolite repression was found only at the lowest *o*-xylene concentrations (3 and 9 μM) tested. At an *o*-xylene concentration of 16 μM, the C23O level was almost the same as that under C limitation. Under O_2-limiting conditions, the C23O levels in response to *o*-xylene were higher than those under C-limiting conditions (Fig. 2b).

Effect of the dilution rate on the expression of BADH and C23O under C limitation. The activities of C23O and BADH were also measured under C limitation at a dilution rate of 0.75 h^{-1}. The BADH and C23O levels were found to be only 3 and 10%, respectively, of those measured at a dilution rate of 0.05

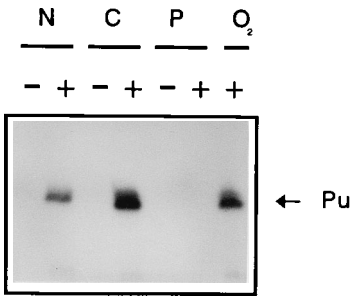

FIG. 3. Level of mRNA derived from the Pu promoter in response to *o*-xylene at $D = 0.05\ h^{-1}$. *P. putida*(pWW0) grown in continuous culture under N, C, P, or O_2 limitation at $D = 0.05\ h^{-1}$ was induced with 3 to 7 μM *o*-xylene. The presence of messengers was analyzed by reverse primer extension of 20 μg of total RNA with the corresponding labelled specific oligonucleotide. The figure shows urea-polyacrylamide gel electrophoresis of the cDNA corresponding to transcripts derived from Pu (134 nucleotides).

h^{-1} (Fig. 4). In this figure, the data obtained at a dilution rate of 0.5 h^{-1} (7) were also included.

Reproducibility of results. The enzyme levels presented in this study are averages of results of enzyme assays done in duplicate or triplicate on two or three cell extracts. These cell extracts were prepared from cells taken from the same steady-state culture. Double standard errors of the individual enzyme assays are shown in Fig. 2 and 4. For each limitation, the independent chemostat experiments at different *o*-xylene concentrations produced consistent results, indicating that the effect of the nutrient limitation on the inducibility of the TOL pathway was statistically significant. The same consistency was observed for the yield values included in Table 1; the yield values of the separate steady-state cultures supplied with different *o*-xylene concentrations were similar for each nutrient limitation (Table 1).

DISCUSSION

We have compared the inducibility of the TOL pathway under C-, N-, P-, S-, and O_2-limiting conditions in a chemostat culture with succinate as the sole source of carbon and energy.

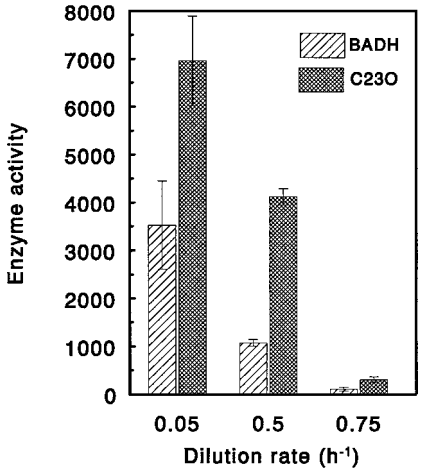

FIG. 4. Influence of the dilution rate on the BADH and C23O activities in response to 2 to 3 μM *o*-xylene during growth on succinate under carbon-limited conditions. Activities are expressed as milliunits per milligram of protein. The errors bars indicate double standard errors of the mean.

The use of the nonmetabolizable inducer *o*-xylene (1) allowed us to maintain a constant concentration of the effector without altering the carbon and energy status of the cells. In all experiments, the dilution rate in the chemostats was kept constant at 0.05 h^{-1}. As the biomass concentration remained relatively constant in all of the chemostat experiments presented, we may assume that the growth rate of the cells was also approximately 0.05 h^{-1}, which is 5% of the maximal growth rate on succinate mineral medium (0.96 h^{-1} [7]).

For all five conditions of limitation, three independent chemostat cultures were run at different *o*-xylene concentrations. In agreement with previous reports (7), the levels of the upper pathway enzyme BADH in cells grown under succinate-limited conditions in the presence of *o*-xylene were high. The BADH levels were between 3,000 and 4,000 mU mg of protein^{-1} at all three concentrations of *o*-xylene tested (3, 6, and 21 μM). Apparently, the binding affinity of the XylR protein for *o*-xylene is sufficiently high for maximal induction at 3 μM. Cells grown in a C-limited chemostat ($D = 0.05\ h^{-1}$) with toluene as the sole source of carbon and energy showed similar BADH levels (2,000 to 3,000 mU mg of protein^{-1}) (results not shown). These levels are the highest reported for *P. putida*(pWW0), and we assume that they are the maximal expression levels that can be reached in *P. putida*(pWW0) at a growth rate of 0.05 h^{-1}. Release of catabolite repression under carbon-limited conditions—the potentially repressive substrate is present at a growth-limiting concentration—is a generally occurring phenomenon (10).

In comparison with these high levels of induction, the expression of the upper pathway in response to *o*-xylene was, in fact, lower under all other conditions of limitation tested. The lowest expression levels of the upper pathway were found under P and S limitation (more than a 40-fold repression). A similar low response to *o*-xylene was found under maximal growth rate conditions in a phauxostat culture, where the residual concentration of succinate was 4 mM (7). Under N limitation, the repression of the upper pathway was, on average, weaker but significant (10- to 50-fold repression). A partial release of catabolite repression under N limitation of a carbon-catabolite repression-sensitive enzyme system was observed earlier (27). Biochemically, this partial release of catabolite repression might have some relationship to the strong links known to exist between nitrogen and carbon metabolism in the cell. Current experiments (unpublished data) indicate that the low inducibility of the TOL upper pathway under P, S, and N limitation can be retraced only partly to the presence of growth-saturating concentrations of succinate. Also when toluene is the sole carbon and energy source, the expression of the upper pathway is lower under P, S, and N limitation than expression under toluene-limited conditions, although the differences are smaller.

Analysis of mRNA showed that the low expression levels of BADH under P, S, and N limitation were correlated to lower transcript levels of Pu, which indicated that catabolite repression of the upper pathway was due to a blockage at the transcriptional level. However, the repression observed in Pu mRNA transcription during N limitation cannot account fully for the low level of BADH activity observed under these conditions (4-fold repression of mRNA levels versus 10-fold repression of BADH levels). A detailed analysis of posttranscriptional effects is required to clarify this apparent discrepancy.

Expression levels of the *meta* cleavage pathway followed qualitatively the same pattern as that of the upper pathway; the lowest expression was found under P and S limitation, and a somewhat higher expression was found under N limitation. The differences in the expression levels of the *meta* cleavage

pathway measured under C limitation were, however, less extreme in comparison to those of the upper pathway. Interpretation of those data is difficult because expression of the *meta* cleavage pathway may proceed through two parallel mechanisms (Fig. 1). The presence of an effector (like *o*-xylene) of the XylR regulatory protein may give rise to an enhanced transcription from the promoter of *xylS* (Ps), resulting in higher levels of the XylS regulatory protein, which in turn may cause transcription from the *meta* pathway promoter (Pm) in the absence of a specific XylS effector. Alternatively, or concomitantly, the inducing activity of the present XylS protein may be enhanced by the binding of an activator like benzoate or toluate (Fig. 1). Theoretically, the induction of the *meta* pathway by *o*-xylene proceeds solely through the first mechanism because no activator of the XylS protein is present in the growth medium and *o*-xylene cannot be converted through the upper pathway. In practice, however, it is very probable that low levels of *m*- and *p*-toluate are present as a result of conversion of traces of *m*- and *p*-xylene (0.5% as analyzed by gas chromatography) (results not shown) that are present as contaminants in the *o*-xylene. The relatively high levels of C23O activity (up to 14,000 mU mg of protein^{-1}) found during O_2 limitation might be attributed to this effect. An alternative explanation could be a lower rate of C23O inactivation, a process in which molecular oxygen is known to be involved (5). The Ps transcript levels under C and O_2 limitation were similarly high (results not shown). Quantification of the intensity of the autoradiograph bands, however, was not accurate enough for us to notice differences in transcript levels within a factor of two. The mRNA data further showed that transcription from Ps under N, P, and S limitation is under the control of catabolite repression (data not shown). To determine if transcription from Pm is also under catabolite repression control under the different limitations tested, similar chemostat experiments should be carried out with a nonmetabolizing inducer of the lower pathway like 2,3-dimethylbenzoate.

On the basis of the presently available data, we cannot pinpoint one particular growth parameter responsible for the extent of catabolite repression. The observation that full catabolite repression occurs not only during nonlimited growth (7) but also in cells growing at only 5% of the maximal rate excludes the growth rate as an important determinant. The relatively high expression levels of BADH and C23O observed under O_2 limitation (20 to 35% and 200 to 300% of the expression levels under C limitation, respectively) indicate that a growth-saturating concentration of succinate (12 to 15 mM) does not automatically cause catabolite repression. Growth-saturating concentrations of a primary carbon and energy source may not even be considered as a prerequisite for catabolite repression, since we also found very low levels of expression of BADH and C23O in response to *o*-xylene under succinate-limiting conditions at the high dilution rate of 0.75 h^{-1} (Fig. 4). The results suggest that the energy status of the cell could be playing a key role. Calculations by Hellingwerf et al. (12) predict that cells of aerobic bacteria grown under conditions where the anabolic substrate is growth limiting (e.g., P, S, and N limitation) have a high energy status, and cells grown under catabolic substrate limitation (C and O_2 limitation) have a low energy status, especially when the carbon and energy source is oxidized relative to biomass, e.g., succinate. Empirical data on the energy status of cells grown under different limitations are scarce. The proton motive force (which is often used as an indicator of the energy status) in cells of *Pseudomonas mendocina* was shown to decrease rapidly in the absence of oxygen (33), which would imply that O_2 limitation may indeed impose an energy limitation on cells of pseudomonads. The relatively low expression levels of the TOL pathway at a dilution rate of 0.75 h^{-1} under C limitation (Fig. 4) may also be explained with this concept: the energy status of the cells under C limitation increases with the dilution rate (12).

The present results provide additional grounds for avoiding nutrient-limiting conditions during the biotechnological treatment of complex wastewaters and polluted soils. P limitation, which often occurs in freshwater habitats (16, 24, 28), especially seems to be an unfavorable condition for expression of a pathway subject to catabolite repression. The relatively high expression levels of the TOL pathway under O_2-limiting conditions does not imply that mineralization of toluene and xylenes will actually occur in the presence of primary substrates; the Michaelis constants for oxygen of purified oxygenases like C23O (0.3 to 2 mg of O_2 liter^{-1} [32]) are generally higher than those reported for the electron transport chain (0.01 to 0.04 mg of O_2 liter^{-1} [17]). Therefore, we assume that the presence of primary carbon and energy sources hampers the degradation of toluene and xylenes not only under N-, P-, or S-limiting conditions but also under O_2-limiting conditions. It remains to be seen to what extent the present data may be extrapolated to other degradation pathways in other strains. The susceptibility of a number of toluene-degrading *Pseudomonas* and *Rhodococcus* strains is currently under investigation.

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