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Carbon Source-Dependent Inhibition of *xyl* Operon Expression of the *Pseudomonas putida* TOL Plasmid

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TOL plasmid-encoded degradation of benzyl alcohol by *Pseudomonas putida* is inhibited by glucose and other compounds related to the main carbohydrate metabolism in *Pseudomonas* species. We report here that this effect is exerted at the level of expression of the *xyl* catabolic operons, and two *xyl* promoters, Pu and Ps, were identified as the primary targets of this inhibition. *xyl* promoter activation was also inhibited by glucose in the heterologous *Escherichia coli* system, apparently not however by the classical mechanism of enteric catabolite repression.

Microbial degradation of aromatic compounds is an important approach to eliminating toxic pollutants from the environment (2, 23, 25). The respective catabolic pathways have generally been analyzed under conditions in which the target compound is present as the sole growth-supporting substrate. In nature, however, mixtures of potential growth substrates are often present, and a more favorable substrate may be degraded to the detriment of the desired degradative activity (19, 21). The phenomenon of certain carbon sources repressing the utilization of less favorable substrates (catabolite repression) has been well described for enteric bacteria (6) and a few genera, such as *Bacillus* (10) and *Pseudomonas* (9, 14). Such regulatory systems have the function of economizing the cellular metabolism. However, they may impede bioremediation of aromatic pollutants in mixtures with more-favorable substrates, as encountered both in situ and in laboratory fermentors in which often, in addition to the aromatic target compound, other more-favorable carbon sources must be fed (cometabolism) (8). Since the degradation of aromatic compounds by *Pseudomonas putida* pWW0(TOL) via the sequential upper and *meta* pathways (encoded by the TOL plasmid-borne *xyl* gene operons) together with the *xyl*-specific induction of these operons has been studied in detail (Fig. 1) (3), this model system was used to analyze the impact of additional carbon sources on *xyl* operon expression.

Effect of additional carbon sources on benzyl alcohol-induced activation of *xyl* promoters in *P. putida*. We observed that the degradation of benzyl alcohol (BA) by *P. putida* pWW0(TOL) was inhibited in the presence of glucose (data not shown). To test whether this effect was reflected in a concomitant inhibition of *xyl* operon expression, β-galactosidase activities were determined (17) in TOL plasmid-cured strains of *P. putida* KT2442, each carrying, integrated into the chromosome, the *xylR* and *xylS* regulatory genes as well as one of the four *xyl* promoter::*lacZ* fusions (Pr::*lacZ*, Ps::*lacZ*, Pu::*lacZ*, or Pm::*lacZ* [Fig. 1]). Cultures were pregrown for 3 h in M9 minimal medium (20) supplemented with 0.25% (vol/vol) micronutrients (5) and 0.2% (wt/vol) Casamino Acids and containing either succinate or glucose at 1.5% (wt/vol). For induction, 5 mM BA was added, and cultures were left to grow for another 3 h. Table 1 shows that the *xylR* gene promoter Pr (which is not induced by BA [1, 13]) reaches similar β-galactosidase levels in succinate- and glucose-grown cultures, while the upper pathway promoter Pu and the *xylS* gene promoter Ps were both induced well by BA in succinate-grown cells but only poorly (about 15%) in glucose-grown cells. As a consequence of this inhibition of *xylS* gene expression, BA-induced activation of the *meta* pathway promoter Pm was also inhibited in the presence of glucose, since Pm in response to upper pathway substrates (such as BA) is stimulated via a regulatory cascade (Fig. 1): XylR protein plus inducer BA activate the *xylS* gene promoter Ps, and, in turn, the overproduced XylS activator then stimulates the *meta* pathway promoter Pm (3).

To confirm that BA-induced expression of the *xyl* upper pathway and *xylS* gene operons, but not expression of the *xylR* gene, was inhibited by glucose at the level of transcription, RNA prepared by the guanidinium isothiocyanate-phenol method (20) was subjected to primer extension analysis (28): overnight cultures of *P. putida* pWW0(TOL) were diluted in M9 minimal medium containing as a carbon source 10 mM BA alone or 10 mM BA plus 1.5% (wt/vol) glucose. Figure 2 shows that mRNA synthesis from the Pr promoter (a tandem promoter consisting of the two individual promoters Pr_1 and Pr_2) was not affected by the presence of glucose, whereas transcription from the Ps and Pu promoters clearly was inhibited.

***xyl* promoter inhibition in *Escherichia coli*.** Since the regulation of *P. putida xyl* promoters can faithfully be reproduced in *E. coli*, we measured β-galactosidase activities of the four relevant *xyl* promoter::*lacZ* fusions, all borne on the low-copy-number vector pJEL122 (24), in *E. coli* ET8000 (16) bearing the required *xylR* and *xylS* activator genes in *trans* (Table 1). Cultures were grown in M9 minimal medium containing either succinate or glucose at 1.5% (wt/vol), and induction was performed by using 7.5 mM 3-methylbenzyl alcohol (3MBA) instead of BA, as the latter induces only poorly in *E. coli*. β-Galactosidase activities were recorded after 7 h of growth. The data obtained (Table 1) show that in *E. coli* 3MBA-induced activation of the Ps (pAH100), Pu (pRD579), and Pm (pIM1) promoters was inhibited by glucose, whereas expression of the *xylR* gene promoter Pr (pAH120) was not. These findings for *E. coli* parallel those obtained for the *P. putida* system and hint at a similar system of carbon source-dependent promoter inhibition being effective in the two species.

Activation of the Pu promoter in *P. putida* in the presence of various other carbon sources. The impact of a number of

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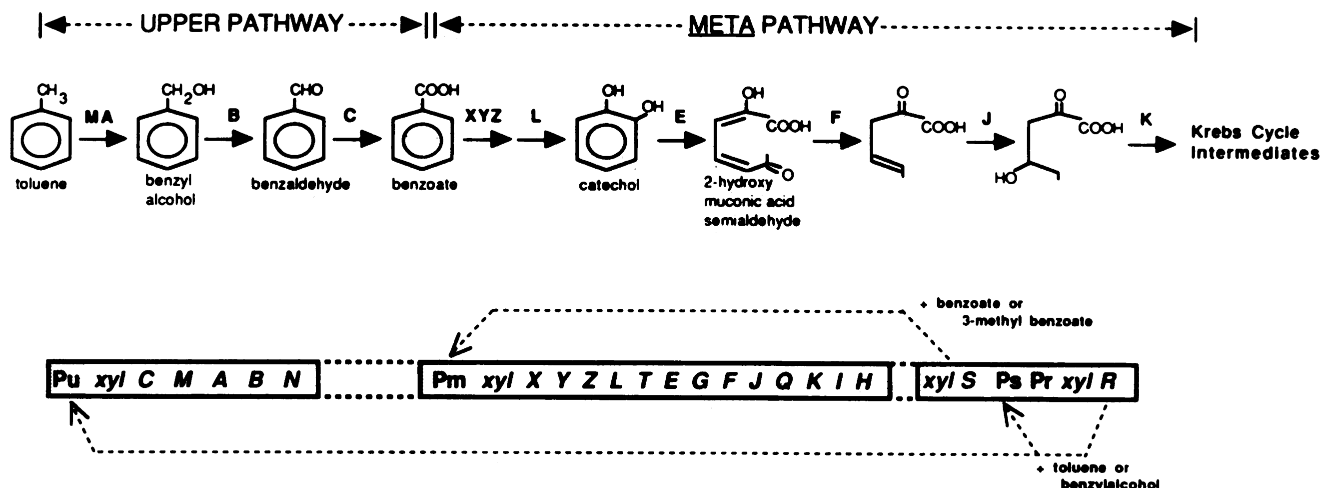

FIG. 1. TOL plasmid-encoded pathway for the degradation of aromatic compounds by *P. putida*. The biochemistry of upper and *meta* pathways and the genetical organization of the corresponding upper and *meta* pathway operons and the *xylR* and *xylS* regulatory genes are shown. The Pr (tandem) and Pm promoters are σ^{70}-dependent standard promoters; the Pu and Ps promoters depend on the specific RNA polymerase σ^{54} subunit. Dashed arrows indicate induction of the Pu and Ps promoters by XylR plus effector (upper pathway substrates) and of the Pm promoter by XylS plus effector (*meta* pathway substrates). Induction of Pm via the regulatory cascade (i.e., by overproduction of XylS resulting from induction of the Ps promoter) does not require *meta* pathway effectors.

different carbon sources on induction of the Pu promoter by benzyl alcohol was determined in TOL plasmid-free *P. putida* KT2442 bearing the *xylR* and *xylS* activator genes and the Pu::*lacZ* fusion in the chromosome. Table 2 shows that succinate, citrate, pyruvate, glycerol, fructose, and arabinose allowed BA-induced activation of Pu as high as that obtained for a culture with only the supplement of Casamino Acids (control), while glucose, gluconate, lactate and acetate inhibited activation of Pu. While the repressive carbon sources represent good substrates for *P. putida* as indicated by the respective growth rates (μ) in Table 2, the converse statement is not true: citrate and succinate are excellent substrates, yet they allow full activation of the Pu promoter. The observed carbon source-dependent inhibition of *xyl* promoter induction therefore probably does not constitute a typical example of bacterial catabolite repression.

Activation of the *xylS* gene promoter Ps in *E. coli cya* and *crp* mutants. Since activation of *xyl* promoters was inhibited by glucose in *E. coli*, the question of whether this effect was mediated by the classical enteric system of catabolite repression arose (6). If this were the case, *xyl* promoters sensitive to inhibition by glucose would require adenylate cyclase (encoded by *cya*) and cyclic AMP receptor protein (encoded by *crp*) for activation in the absence of glucose. To test this possibility, plasmid pUJ100 (identical to plasmid pAH100 [11] but carrying apart from the Ps::*lacZ* fusion the *xylR* regulatory gene in *cis*) was introduced into a series of isogenic *E. coli* wild-type, *cya*, and *crp* mutant strains (strains JWL184-1, JLV8, and JLV25, respectively [26]), and 3MBA-induced β-galactosidase

TABLE 1. Activation of *xyl* promoter::*lacZ* fusions

Organism and carbon source[a]	β-Galactosidase activity (U) for *xyl* promoter::*lacZ* fusions with (+) or without (−) inducer[b]							
	Pr::*lacZ*		Ps::*lacZ*		Pu::*lacZ*		Pm::*lacZ*	
	−	+	−	+	−	+	−	+
P. putida[c]								
Succinate	250	220	30	820	40	1,970	20	780
Glucose	280	250	40	120	50	300	10	30
E. coli[d]								
Succinate	330	360	50	1,400	110	6,310	170	2,350
Glucose	540	570	50	330	90	1,530	70	240

[a] Carbon sources were added at 1.5% (wt/vol).
[b] As inducer, 5 mM BA or 7.5 mM 3MBA was applied to assays of *P. putida* or *E. coli* strains, respectively.
[c] *xyl* promoter::*lacZ* fusions were integrated into the chromosome of strain KT2442 *xylR/S* (see the text).
[d] The Pr, Ps, and Pu promoter::*lacZ* fusions (on plasmids pAH120 [11], pAH100 [11], and pRD579 [7], respectively) were assayed in strain ET8000 (16) bearing *xylR* in *trans* on plasmid pTS174 (13); the Pm promoter::*lacZ* fusion (plasmid pIM1, carrying Pm plus 2 kb of promoter upstream region) was assayed in strain ET8000 bearing *xylR* and *xylS* integrated into the chromosome.

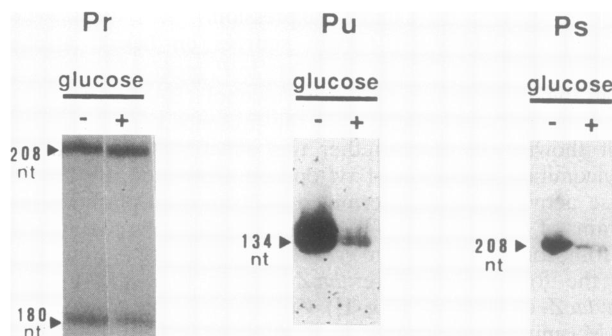

FIG. 2. Effect of glucose on the synthesis of mRNA from *xyl* gene promoters in *P. putida* pWW0(TOL). mRNA extracted from cultures containing as a carbon source only BA (−) or BA plus 1.5% glucose (+) was subjected to primer extension analysis with primers complementary to the Pr, Pu, and Ps promoter downstream regions, namely 5′-ACGGATCTGGCTGCTAAGGTCTTGC-3′, 5′-GGCCAGCGTCACAGACTCCAGGCG-3′, and 5′-CGGAGACTGCATAGGGC-3′, respectively. Extended cDNA products were analyzed on urea-polyacrylamide sequencing gels. nt, nucleotides.

TABLE 2. Activation of the upper pathway promoter Pu in *P. putida* KT2442 *xylR/S*

Carbon source[a]	Growth rate[b]	β-Galactosidase activity (U) for Pu::*lacZ* fusion with (+) or without (−) inducer[c]	
		−	+
Succinate	0.49	60	1,830
Citrate	0.58	60	1,740
Pyruvate	0.27	50	1,700
Arabinose	0.30	100	1,850
Fructose	0.31	80	1,290
Glycerol	0.28	70	1,550
Glucose	0.43	40	620
Gluconate	0.48	80	600
2-Ketogluconate	0.30	80	470
Acetate	0.58	40	420
Lactate	0.34	50	300
Casamino Acids	0.99	80	1,780

[a] Carbon sources were added to minimal medium at 0.8% (wt/vol) except for the control, which contained only Casamino Acids at 0.2% (wt/vol).

[b] Specific growth rates (μ [hour^{-1}]) were determined from exponentially growing cultures without BA.

[c] BA, used as an inducer, was added at 5 mM.

activities were determined for cultures grown in M9 minimal medium, as described above, supplemented with succinate. The data obtained showed that in these cultures, i.e., in the absence of glucose, transcription from the 3MBA-induced *xylS* gene promoter Ps does not require the cyclic AMP receptor protein, because β-galactosidase activities for uninduced and induced cultures (without and with 3MBA, respectively) in *crp* mutant JLV25 (110 and 950 U, respectively) were as high as those in the wild-type JWL184-1 (100 and 820 U, respectively). Since the *cya* mutant JLV8 (180 and 530 U, respectively, for uninduced and induced cultures) still exhibited about 65% of the wild-type activity, adenylate cyclase does not seem to be essential for activation of Ps.

Carbon source-dependent *xyl* promoter inhibition in *P. putida* thus apparently does not follow the classical system of enteric catabolite repression involving cyclic AMP as the signal for glucose starvation. This is in agreement with earlier studies excluding a role of cyclic AMP in carbon regulation in *Pseudomonas* species (18, 22). The finding that not one particular most-favorable carbon source but rather a number of compounds related to the primary carbohydrate metabolism of *Pseudomonas* species evoke *xyl* promoter inhibition renders it unlikely that a specific repressor protein directly responding to the respective repressive substrate (as in the case of succinate repression in *Pseudomonas aeruginosa* [15] or glucose repression in *Bacillus subtilis* [10]) mediates the observed inhibitory effect. Instead, another unidentified regulatory system present in both *P. putida* and *E. coli* probably responds to a metabolite or physiological condition related to the active primary carbohydrate metabolism, thus leading to the observed promoter inhibition. A number of mechanisms that could potentially bring about this effect can be conceived, ranging from changes in promoter DNA supercoiling, which may affect gene expression in response to nutritional changes (4, 12), to the notion that the two identified target promoters directly susceptible to carbon source inhibition, namely Pu and Ps, both require the specific RNA polymerase subunit σ^{54} for transcription (3). It may thus be that specifically σ^{54}-dependent promoters either directly (e.g., by supercoil effects distorting essential activator upstream binding sites characteristic for σ^{54}-dependent promoters [27]) or indirectly (e.g., via modulation of synthesis of σ^{54}) are negatively affected in the presence of repressive carbon sources. Future work will aim at identifying the molecular basis of the described inhibition of *xyl* promoters both in *P. putida* and in the heterologous *E. coli* background.

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